The Central Amygdala:

An Electrophysiological Study

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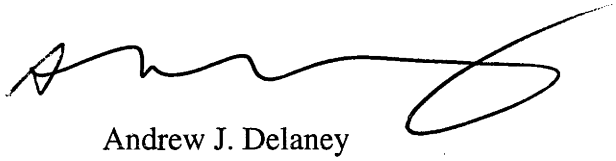
Declaration

I hereby declare that the research embodied in this thesis is original and was completed solely by the author.



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PUBLICATIONS ARISING FROM DATA PRESENTED IN THIS THESIS

PAPERS APPEARING IN REFEREED JOURNALS

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A.J. Delaney and P. Sah (2000) Pathway specific targeting of $GABA_A$ receptor subtypes to somatic and dendritic synapses in a central neurone (Submitted).

CONFERENCE ABSTRACTS

A.J. Delaney and P. Sah (1998) Synaptic transmission in the central amygdala, *Proceedings of the Australian Neuroscience Society*, Vol 9.

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ABSTRACT

The amygdala is a multinucleate body located deep in the temporal lobe. Part of the limbic system, the amygdala has been shown to be a crucial region in the neural processes underlying emotion. The central nucleus of the amygdala is the output nucleus for the amygdala, and is believed to be maintained under a high level of inhibition from a network of inhibitory neurones contained within a larger structure called the central extended amygdala. This thesis investigates the synaptic physiology of neurones of the central amygdala. This study has utilized whole cell patch pipette recording methods to record the currents underlying excitatory and inhibitory synaptic responses in these neurones and a range of pharmacological agents to determine the receptors mediating these currents.

Excitatory transmission in the central amygdala was found to be mediated by the activation of glutamate receptors. These receptors were found to be of the AMPA and NMDA types, with sensitivity shown to the antagonists CNQX and D-APV respectively. Inhibitory synaptic currents in these neurones were found to result from activation of GABA receptors. Two types of GABA receptors were identified according to sensitivity to the antagonist bicuculline, both contributing to synaptic currents. The first type was a typical $GABA_A$ receptor that displayed sensitivity to low concentrations of bicuculline and was positively modulated by 1,4-benzodiazepines. The second type was an atypical GABA receptor that had pharmacological properties consistent with $GABA_C$ receptors found in retina (insensitivity to low concentrations of bicuculline, but sensitivity to the antagonist TPMPA) and properties normally associated with particular subtypes of $GABA_A$ receptors (sensitivity to 1,4-benzodiazepines and barbiturates). The specific effect of the 1,4-benzodiazepine was unlike any previously reported in that the currents mediated by these atypical GABA receptors were partially blocked by these agents. These receptors also displayed reduced sensitivity to barbiturates and insensitivity to the anaesthetic propofol when compared to typical $GABA_A$ receptor mediated currents.

The synaptic distribution of these two GABA receptor types was investigated by studying the sensitivity of evoked and spontaneous inhibitory synaptic currents to the antagonists bicuculline and TPMPA. The latter events indicated that the predominantly activated synapses are located on the dendritic regions of these neurones and contain a mixture of both types of GABA receptor. These synaptic events could be evoked electrically by stimulating in the region of the intercalated cell masses, positioned laterally to the central amygdala, or as part of a disynaptic circuit from the basolateral amygdaloid complex. A population of large spontaneous events that occur at very low frequency in control conditions were found to be mediated by $GABA_A$ receptors only. These synaptic responses could be evoked electrically medially to the central amygdala.

These experiments show that there exists a novel type of GABA receptor in the central amygdala that is insensitive to bicuculline, sensitive to TPMPA and negatively modified by the classical 1,4-benzodiazepines. Secondly, these novel receptor types are selectively targeted to particular synapses in the neurone, in a pathway dependant manner. The third finding is that the lateral division of the central amygdala receives inhibitory input following the flow of input through the amygdala from the lateral to medial parts, as well as medial to lateral inhibition from as yet unidentified sources.

LIST OF ABBREVIATIONS

ANATOMICAL

B	basal nucleus of meynert
BLA	basolateral amygdala
BLC	Basolateral Amygdaloid Complex
BMA	basomedial amygdala
BNST	bed nucleus of the stria terminalis
CA1	hippocampus, CA1 region
Ce	central amygdala
CEA	Central Extended Amygdala
CeI	central amygdala, intermediate division
CeL	central amygdala, lateral division
CeLC	central amygdala, lateral division, capsular region
CeM	central amygdala, medial division
EC	external capsule
ICM	intercalated cell masses of the amygdala
LA	lateral amygdala
lab	longitudinal association bundle
MeA	medial amygdala
NLOT	nucleus of the lateral olfactory tract
SI	sublenticular substantia innominata

RECEPTORS, LIGANDS AND DRUGS

AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
BIC	bicuculline (methiodide salt)
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
D-APV	DL-2-amino-5-phosphonovalerate
GABA	γ-aminobutyric acid
$GABA_A$	γ-aminobutyric acid receptor, type A

$GABA_B$	γ-aminobutyric acid receptor, type B
$GABA_C$	γ-aminobutyric acid receptor, type C
Gly	glycine
KYN	kynurenic acid
NMDA	*N*-methyl-D-aspartate
PTX	picrotoxin
STR	strychnine
TPMPA	1,2,5,6-tetrohydropyridine-(4-yl)methylphosphinic acid
TTX	tetrodotoxin

ELECTROPHYSIOLOGICAL

CsGluc	cesium gluconate
E_{Cl}	equilibrium potential for chloride ions
EPSC	excitatory post synaptic current
I	current
IPSC	inhibitory post synaptic current
$KmeSO_4$	potassium methylsulfate
mIPSC	miniature spontaneous inhibitory post synaptic current
mEPSC	miniature spontaneous excitatory post synaptic current
Vm	membrane potential

CONTENTS

1. INTRODUCTION

1.1. THE BRAIN

The brain, like all other organs of the body, is an organisation of differentiated cells that perform specific functions within the processes required to sustain the life of the body. What sets the brain apart from other organs, is the degree of specialisation of cells within the brain, the organisation of these units into multiple functional modalities, the complexity of the functions that this cellular organisation achieves, and the degree by which the brain interacts with and indeed presides over the other functions of the body.

The brain and nervous system are composed of billions of specialised cells called neurones, accompanied by even more non-neuronal support cells called neuroglial cells. Neurones have evolved into highly organised cellular structures, which are capable of conducting electrical signals, often over very large distances, and communicating with each other at specialised structures called synapses. Some neurones also communicate with non-neuronal cells, such as muscle fibres and endocrine cells by releasing substances onto these cells at specialised junctions, such as the neuro-muscular junction. Neurones are organised into patterns of communication called circuits, which link particular subsets of neurones into a functional unit. These communicational patterns also direct the anatomical distribution of those neurones into groups called nuclei. The interaction of these nuclei through electrical communication of neurones within them are the basis of brain function.

The functions of the nervous system are generally classified into sensory, motor, and behavioral aspects. Sensory functions receive and process information about the internal and external environment of the body. Functional modalities within this group include the visual system, auditory system, olfactory system, vestibular system, and the somatosensory system. Motor functions control the movement of the

body through coordinated innervation of muscle. The neuronal mechanisms underlying the behavioral functions of the brain are less understood than sensory and motor functions. These functions include cognition, emotion, memory and consciousness and involve specialised systems such as the association cortex, the limbic system and the autonomic nervous system.

1.2. THE LIMBIC SYSTEM AND EMOTION

1.2.1. Historical perspectives

James W. Papez is credited with the original notion that the complex behavioral processes that we collectively coin in the word 'emotion' occur in the circuitry of a group of nuclei, in the limbic lobe. This lobe, first described by the french anatomist Broca, was originally described as the cerebral cortex rimming the corpus collosum on the medial aspect of the hemispheres. This includes the cingulate and hippocampal gyri (and the isthmus which connects them), and various gyri associated with the olfactory system. Evidence accumulated that the hypothalamus was intimately involved in the expression of emotion. Papez reasoned that emotion was a process that occurred in consciousness and that higher cognitive processes affect emotion. Thus the neural system that formed emotion must connect higher cortical centres involved in cognitive function with the hypothalamic region where emotional expression occurred. He proposed a neural circuit which included the mammilary body, the anterior nuclei of the thalamus, the gyrus cinguli and the hippocampus, which 'may elaborate the functions of central emotion as well as participate in emotional expression' (Papez, 1937).

Just two years after Papez had published his proposed emotion circuit Kluver and Bucy published a study describing the effects of temporal lobotomy in rhesus monkeys (Kluver & Bucy, 1939). In this study, the authors noted that among the effects of the temporal lobotomy were 'marked changes to emotional behavior or

absence of emotional reactions in the sense that the motor and vocal reactions generally associated with anger and fear are not exhibited'. These behavioral effects (later to become known as the Kluver-Bucy syndrome) led Paul MacLean to expand the emotion circuit proposed by Papez, to include hypothalamic regions, the septal area, nucleus accumbens, orbitifrontal cortex, and the amygdala (MacLean, 1949). This expanded version of Papez's circuit has now become known as the limbic system.

1.2.2. Limbic Structures participate in emotional processes

The modern view of the limbic system is a complex neuronal circuit composed of extensive direct and indirect connections between various cortical areas, cingulate gyrus, hippocampus, thalamus, amygdala and hypothalamus.

The hypothalamus acts on the endocrine system and the autonomic nervous system to control a variety of regulatory systems. It contains several classes of peptidergic neuroendocrine cells that control the endocrine system by releasing neuroendocrine products either directly into general circulation via the posterior pituitary or indirectly via the portal circulatory system in median eminence. The hypothalamus also functions as the head ganglion of the autonomic system. It sends neuronal outputs to the brainstem and spinal cord that innervate preganglionic autononic neurones. Stimulation of the hypothalamus has been shown to be able to produce all of the autonomic and somatic effects of emotional behavior, even in the absence of all other parts of the limbic system. The role of this structure is thus considered to be the point of convergence of the processes occuring in the limbic system which activates the autonomic and somatic responses associated with emotional response.

Limbic structures that provide input to the hypothalamus include the amygdala and the hippocampus. The hippocampus has been intensely studied in recent years as a site where processes of memory and learning are though to occur. It is well established that hippocampus integrates and relays cortical information to the hypothalamic region via substantial connections passing through the fornix, however its role in the formation and expression of emotion is not well understood.

In contrast, the amygdala has been shown to be a critical structure in emotional processes. Lesioning the amygdala produced marked effects on emotional behavior – particularly fear (Rosvold *et al*, 1954). Projections from the amygdala to thalamic and brainstem regions have been demonstrated anatomically (Otterson, 1980; Otterson, 1981) and functionally (Clugnet *et al*,1990), as have connections with the limbic corticies (Pascoe & Kapp, 1987).

Various studies have shown that lesions or ablations of the limbic corticies – the orbitofrontal cortex, the cingulate gyrus and cortical regions of the temporal lobe have effects that alter emotional responses (Kluver & Bucy, 1939). Prefrontal lesioning (frontal lobotomy) reduced aggression in primates and was used for a short time as a treatment for severely mentally ill humans to reduce anxiety. Furthermore, stimulation of prefrontal cortex produces a range of autonomic responses, indicating association with hypothalamic or brainstem regions (Galsema *et al*, 1987). The lesioning of the temporal portion of limbic association cortex also resulted in emotional abnormalities. Stimulation in this area produces emotional expression particularly feelings of fear. These regions have also been shown to project into the amygdala and hippocampus.

1.1.1. What is emotion?

The concept of the limbic system indroduced by Papez and later expanded by MacLean, invoked an ideal that emotions were the net result of neuronal processes occuring within this system. However, the centralization of all emotional processes to a defined circuitry within the limbic system has not been demonstrated, and this concept of a dedicated emotional system is faultering under increasing scrutiny (LeDoux, 2000; Rogan & LeDoux, 1996).

The question 'what is emotion' was posed in the late nineteenth century by William James (James, 1884) but has remained unanswered now for over a century. Despite the fact that our own experience of emotions allows us to know intuitively what they are, no scientific definition of emotion has become accepted (LeDoux, 1995; Simonov, 1997). Descriptions of emotion are also vague and non-committal, which perhaps arises from the varied and subjective nature of emotion and our inability to

rationalise a feeling into a measurable, definable entity. Emotion has been described as a 'short label for a very broad category of experiential, behavioural sociodevelopmental, and biological phenomena' (Cacioppo & Gardner, 1999), or consisting of two dimensions where on one axis emotional states range from positive (happy) to negative (fearful or angry), and the other axis low to high arousal states (Gallagher & Chiba, 1996). Alternately, some theorists have tried to catagorise emotion into basic emotions which in combination give rise to more complex emotions (Izard, 1992), while others have argued that even these 'basic emotions' may not be emotions at all (Ortony & Turner, 1990).

An alternative to trying to define emotion as a neurological entity, is to describe different expressions of emotion, such as such as guilt, embarrassment, anger, happiness or fear, and to relate them and even measure them according to a range of physical manifestations of these emotional states. By studying these narrow models of emotion it is thought that common mechanisms of all emotions may be ascertained, and the organization of all emotional circuitry established (LeDoux, 1995).

The most commonly researched and best-understood expression of emotion is fear (LeDoux, 1996). This understanding has come from the study of emotional disorders in humans related to the regulation of fear, anxiety, phobia, panic and post-traumatic stress, and the relation of the physiological manifestations of these disorders in humans to behavioural measures that can be obtained from animal models of fear. Though no absolute evidence exists that any animal model of fear is actually producing fear as experienced by humans, the behavioural correlates and physiological determinants studied are common across mammalian species in similar experimental fear paradigms (LeDoux, 1996). These studies have allowed researchers to examine the neuronal pathways involved in the control of fear reactions, and perhaps in the formation of the emotion itself, rather than studying the emotion on subjective grounds. They have demonstrated that at least in the case of this emotion, it is not only the regions of the brain previously included in the limbic system which have a role (LeDoux, 2000).

1.2.4. Fear conditioning

The study of fear most commonly involves the use of a behavioural model of fear called fear conditioning. This learning paradigm is a development from the observations of Pavlov, whose work on salivation in dogs in response to visual and acoustic stimuli led to the model of classical conditioning where conditioned stimuli (CS) are paired with unconditioned stimuli (US) (Pavlov, 1928). One of the conditioning models developed by Pavlov – defense conditioning, has now become known as fear conditioning.

The simplest form of fear conditioning involves the pairing of an innocuous CS, such as an auditory or a visual stimuli, with a noxious US such as a footshock. After just a few such pairings, or even a single pairing, behaviour consistent with defense and fear is exhibited in response to either the CS, the US or both.

Perhaps the most valuable findings arising from the use of fear conditioning in behavioural studies arose from studies using selective ablation of particular regions of the brain to disrupt the acquisition or expression of the conditioned state. In particular, lesioning studies of the amygdala, which had been shown to decrease responses to threatening experiences in monkeys (Rosvold *et al.*, 1954) and cats (Schreiner & Kling, 1956), were shown to block fear conditioning (Davis *et al.*, 1993; LeDoux *et al.*, 1990; Sananes & Davis, 1993), or the expression of the fear conditioned state after acquisition (Davis *et al.*, 1993; Hitchcock & Davis, 1991; Hitchcock & Davis, 1986).

A number of axonal tracing studies (Kapp *et al.*, 1985; Kapp *et al.*, 1985; LeDoux *et al.*, 1990; McDonald, 1991; McDonald, 1991; Ottersen, 1980; Ottersen, 1981; Ottersen, 1982; Ottersen & Ben-Ari, 1979) and electrophysiological studies (Clugnet *et al.*, 1990; Ono *et al.*, 1995) have identified the sources of input to the amygdala and targets of amygdala output, and have led to the formation of a putative fear conditioning circuit. This circuit (Figure 1.1) places the amygdala at a position where processed and unprocessed sensory information is received, and after presumably some integration of this information has occurred, output is sent to an

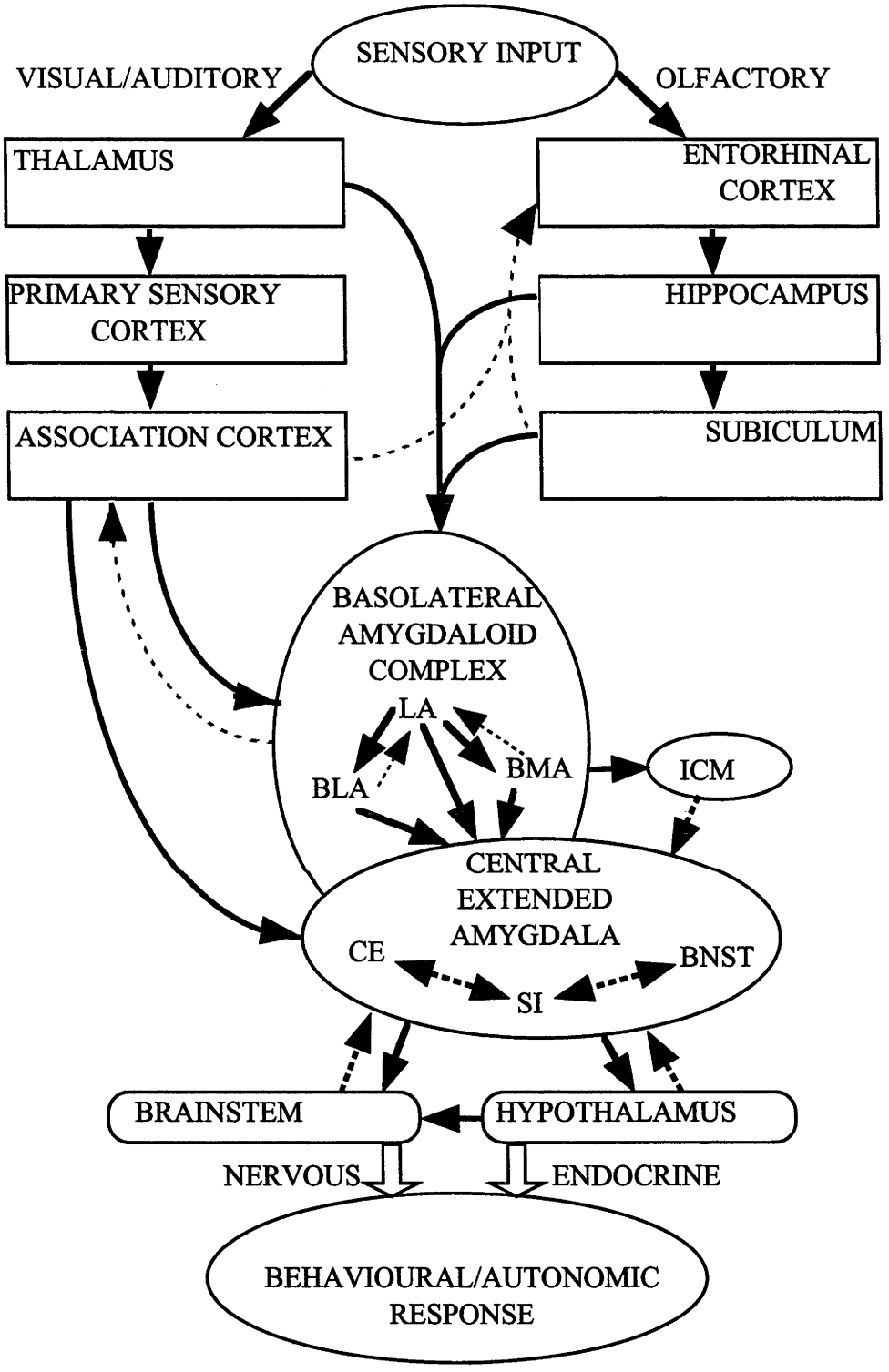

Figure 1.1. Schematic of proposed neuronal circuits involved in emotions (adapted from [LeDoux,1993; Charney,1996]).
Major excitatory pathways indicated by heavy unbroken arrows, major inhibitory pathways indicated by heavy broken arrows and minor/indirect inhibitory pathways indicated by light dashed arrows.

effector system in the hypothalamus or brainstem. Furthermore, the amygdala has now become widely accepted as the region of the brain where this form of associative learning occurs (Armony & LeDoux, 1997; Davis, 1992; Davis, 1994; Faneslow & LeDoux, 1999).

1.3. THE AMYGDALA

The amygdala is a complex of connected nuclei situated medially in the temporal lobe. In the primate, cat and rat, it comprises of four main nuclei; Lateral (LA), Basolateral (BLA), Basomedial (BMA) and Central Amygdala (Ce), several smaller accessory nuclei, and an associated area of cortex (Figure 1.2.). The four main nuclei are also composite of smaller divisions, according to connectivity and cytoarchitectonic distinctions. Although nomenclature across the species is somewhat divergent, corresponding amygdaloid nuclei and connectivity between these nuclei has generally been described (Paré & Smith, 1998).

Anatomically, amygdaloid nuclei are grouped into the laterally situated Basolateral Amygdaloid complex (consisting of the LA, BLA and BMA), the medially situated Central Extended Amygdala (consisting of the Ce, the lateral bed nucleus of the stria terminalis (BNST) and the sublenticular substantia innominata (SI) (Sun *et al.*, 1994). The overlying dorsolateral region of cortex contains neurones that closely resemble those of the basolateral complex (McDonald, 1992). Furthermore, several cortical inputs to the basolateral complex also project to this region, which then projects to basolateral and central amygdaloid neurones (Turner & Zimmer, 1984). For these reasons, these cortical regions are often referred to as the cortical nuclei of the amygdala (McDonald, 1992).

Separating the cortical nucleus and basolateral complex is a dense fibre bundle called the external capsule (EC) and a similar but somewhat smaller bundle between the basolateral amygdaloid complex and the central nucleus is called the longitudinal association bundle (lab) (Figure 1.2.). Lying within these dense fibre

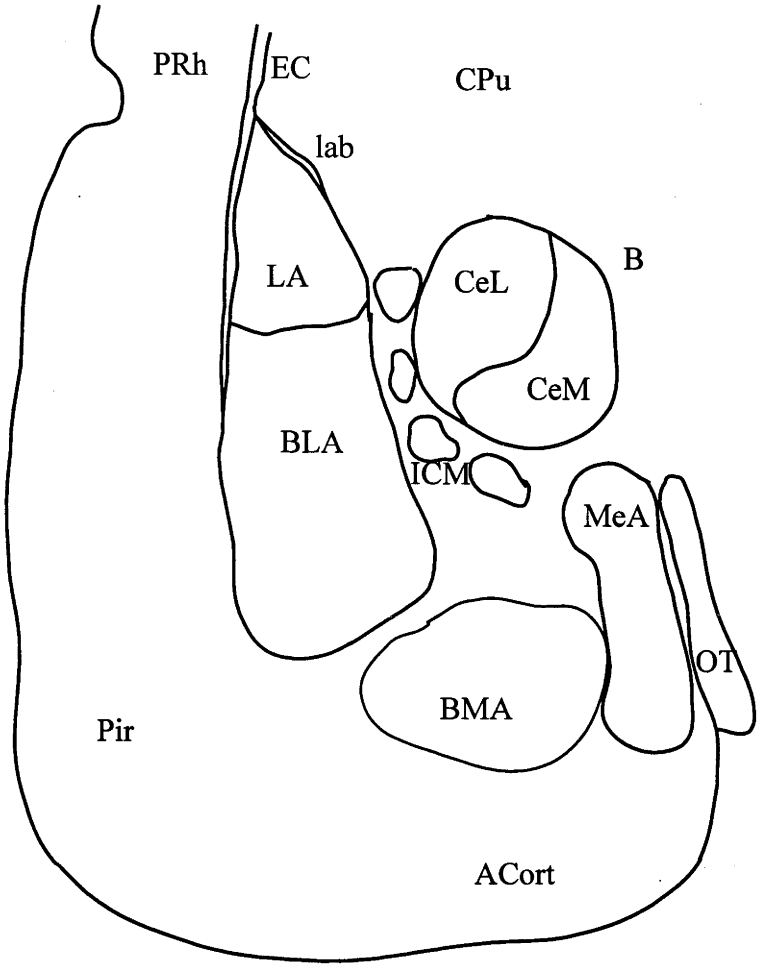

Figure 1.2. Schematic diagram of the rat amygdala in coronal section. Abbreviations- ACort - cortical nucleus of the amygdala, B - basal nucleus of meynert, BLA - basolateral amygdala, BMA - basomedial amygdala, CeL - central amygdala, lateral division , CeM - central amygdala, medial division, CPu - caudate putamen, EC - external capsule, ICM - intercalated cell masses, LA - lateral amygdala, lab - longitudinal association bundle, MeA - medial amygdala, OT - optic tract, Pir - piriform cortex and PRh - perirhinal cortex.

tracts are heterologous clumps of neurones called the intercalated cell masses (ICM) (Millhouse, 1986).

1.3.1. The Basolateral Amygdaloid Complex

The basolateral amygdaloid complex has been shown in a variety of species to contain two principle types of neurones (McDonald, 1992) – a pyramidal like neurone, densely covered in spines, and a stellate neurone with an oval somata and very few spines. The pyramidal type is the predominate type of neurone in the complex, and although its size does vary across nuclei these cells are larger than the stellate cells. Morphologically similar to cortical pyramidal neurones, the amygdaloid pyramidal neurones contain glutamate and project out of their nuclei. The smaller and less prominent stellate cells contain gamma-amino butyric acid (GABA) and neuropeptides, and generally project locally within their nuclei (Davis *et al.*, 1994). Though cell size has been used to identify the boundaries of LA, BLA and BMA, these regions largely contain homogenous populations of cell types. As such other aspects, such as cell density, connectivity and immunolabelling are also used for defining divisions within these nuclei.

The LA is the most dorsal nucleus in the rat basolateral amygdaloid complex along the entire rostrocaudal extent of the complex. It lies between the external capsule on its lateral aspect and the intermediate capsule medially. Based on cell density, immunoreactivity to the enzyme acetylcholine esterase (AChE), timm staining, and connectional data, the rat LA has been divided into dorsolateral, ventrolateral, and medial divisions (Savander *et al.*, 1996; Turner & Zimmer, 1984).

The BLA (also referred to as the basal nucleus) is the largest and most complex nucleus of the rat basolateral amygdaloid complex. It lies ventrally to the LA for the most part between EC and IC. Though the border between LA and BLA is not clearly defined by fibres, the BLA can be distinguished from LA by the larger size of its neurones and its darker appearance under the light microscope. In all species

studied, the BLA contained the largest amygdaloid neurones (Price *et al.*, 1987). Using similar histochemical and cytological methods, Turner and Zimmer describe five subdivisions in the rat BLA – anterior, ventral, middle, medial, and posterior parts, with the middle subdivision further divided laterally and medially (Turner & Zimmer, 1984), whereas Savander *et al* subdivide the BLA into three subdivisions – magnocellular, intermediate and parvicellular (Savander *et al.*, 1995). The latter study also incorporated connectional observations in its nomenclature and will be used in the remainder of this thesis.

The BMA (or accessory basal nucleus) is the most ventral nucleus of the basolateral complex, lying in the caudal half of the complex. This region in the cat and primate appear quite homologous, and in these species is clearly subdivided into magnocellular, parvicellular and superficial divisions (Price *et al.*, 1987). In contrast, the rat BMA does not show clear subdivisions. The most homologous region in the divisions of cat and primate is an area similar to the superficial division, which like that of cat and primate, has larger cells and higher AChE immunoreactivity. However, based on connectivity, this region has since been more recently described as part of the amygdalohippocampal area (Savander *et al.*, 1996).

1.3.2. The Central Extended Amygdala

The concept of the central extended amygdala (CEA) arose from initial observations by Johnston (Johnston, 1923) that during development and into the adult, the bed nucleus of the stria terminalis and the centromedial amygdaloid nuclei formed a continuum. This was further demonstrated and broadened to include the sublenticular substantia innominata by de Olmos (De Olmos, 1972). Subsequent studies have shown similarities between the cell types within these regions (Alheid *et al.*, 1998; McDonald, 1992; Shimada *et al.*, 1989).

The central amygdala

The CeA in all mammalian species studied has two prominent divisions based on cell morphology – lateral (CeL) and medial (CeM) (Alheid *et al.*, 1995; McDonald,

1992). In the rat, the CeL has been further divided into two subdivisions– the core region of lateral (CeL) and the lateral capsular division (CeLC), and the CeM has been divided into an anterodorsal region (CeMad), a anteroventral region (CeMav), and postoventral region (CeMpv) based on cytoarchitectural grounds (Alheid *et al.*, 1995; Cassell & Gray, 1989; McDonald, 1982; Price *et al.*, 1987). Others have also included an intermediate division lying between the CeL and CeM based on counterstaining using Nissl and golgi staining methods (McDonald, 1982) and inter- and intra-connectivity of these divisions (Jolkkonen & Pitkanen, 1998).

The CeM contains neurons with ovoid, fusiform or piriform shaped somata. These cells have been grouped into two types based on dendrite morphology – one with sparsely spined thin primary dendrites that branch into spiny secondary dendrites, and one with thick non-spiny dendrites (Price *et al.*, 1987),(Cassell & Gray, 1989),(McDonald, 1982). Axons of CeM neurons project out of the CeM into stria terminalis and the ventral amygdalofugal pathway, with only a small number of local collaterals remaining in the CeM. Thus these neurones have been described as the primary projection neurones of the central amygdala (McDonald, 1982).

The principal cell type in the CeL and CeLC has an ovoid or round soma and medium density of spines which increases on the secondary dendrites. The axons of these neurones collateralise locally onto other medium spiny neurons in CeL, and project out of the CeL to the bed nucleus of the stria terminalis (McDonald, 1982; Sun & Cassell, 1993). These neurons also feature spinelike protrusions on the initial segment, similar to those seen in the striatum and cerebral cortex that have been found to be post synaptic specialisations. Two other cell types have been described in the CeL (but excluded from the capsular region) by Cassell and Gray (Cassell & Gray, 1989). One is a small population of larger neurones with long piriform somata and thick aspinous primary dendrites. Primary dendrites of these neurones either emerge from the apical position, bifurcate at about 10μm from the perikaryon and extend away from the cell in the same direction as the primary dendrite, or emerge basally, bifurcate at about the same distance and radiate out from the cell. The second is a population of smaller ellipsoid shaped neurones with no spines, and short dendrites emerging from the most distal ends of the soma (Cassell & Gray, 1989).

The intermediate division of the CeA is only described in rat, using counterstaining (McDonald, 1982) or connectional data (Pitkänen *et al.*, 1997). The cells of this region were described by McDonald as being very similar to those of CeM, and having dendritic fields overlapping those of CeM. The same study indicated that the axons of these neurones branch locally in the vicinity of the cell. This observation was supported by a later study using anterograde tracing that indicated that the majority of CeI projections were intradivisional (Pitkänen *et al.*, 1997).

Numerous studies have demonstrated that the CeA has a high density of GABAergic neurones (Jongen-Rêlo & Amaral, 1998; McDonald & Augustine, 1993; Nitecka & Ben-Ari, 1987; Pitkänen & Amaral, 1994), and the highest density of GABAergic terminals in the amygdala (Nitecka & Ben-Ari, 1987). The CeA has also been found to contain a high density of cells containing a variety of neuropeptides including substance P (SP), vasoactive intestinal polypeptide (VIP), neurotensin (NT), galanin (GAL), somatostatin (SOM) corticotrophin-releasing factor (CRF) and enkephalin (ENK) (Roberts, 1992). VP, NT, SOM and CRF reactive neurones were found on numerous cell types in both CeL and CeM, whereas SP and GAL reactive neurones were confined to the aspiny ovoid and pyrimaform neurons of CeM (Cassell *et al.*, 1986; Cassell & Gray, 1989; Roberts, 1992).

The bed nucleus of the stria terminalis

Golgi studies have shown that the neurones of the bed nucleus of the stria terminalis (BNST) are identical to corresponding regions in the Ce. The anterodorsal part of lateral BNST contains both the predominate medium spiny and the spine sparse neurones found in the CeL, whereas the ventro posterior parts of lateral BNST contain neurones identical to those of the CeM (Alheid *et al.*, 1995; McDonald, 1992). The BNST has also been shown to produce a number of the neuropeptides found in Ce neurones, including CRF, NT, SP and SOM (Shimada *et al.*, 1989) and to also contain a high density of GABAergic neurones (Nitecka & Ben-Ari, 1987). The medial BNST shows cellular homology to a region medial to the Ce, which is described as medial amygdala in some studies.

Sublenticular substantia innominata

This region lies between the globus pallidus and the lateral proptic-lateral hypothalamic grey region (dorsally to ventrally), merging with the BSTL and BSTM at its rostromedial aspect and the CeM caudolaterally (de Olmos *et al.*, 1985). Neurones of the SI are a homologous population medium sized spindle shaped neurones which have dendritic fields that freely overlap those of the CeM (McDonald, 1982).

1.4. PROJECTIONS TO THE AMYGDALA

Numerous axonal tracing studies have revealed each amygdaloid nucleus receives input from multiple yet distinct brain regions generally in a non-overlapping manner. Based on axonal tracing studies, lesioning studies and electrophysiological recordings, the basolateral amygdaloid complex is believed to be the sensory input station of the amygdala (Davis *et al.*, 1994; Pitkänen *et al.*, 1997). Highly processed sensory information from a variety of cortical and subcortical regions arrives either directly to regions in these nuclei or via the cortical nuclei of the amygdala (Mascagni *et al.*, 1993).

The LA has been shown to receive convergent inputs from thalamic (lateral and medial geniculate nuclei) and secondary cortical areas of the visual and auditory systems (temporal cortex) directly or via the perirhinal cortex (LeDoux, 1993; Ono *et al.*, 1995; Romanski *et al.*, 1993). Somatosensory information from the medial geniculate nucleus, the supra geniculate nucleus and the posterior intralaminar nucleus arrives directly or via the temporal cortex and insular cortex, with somatosensory information from the ventral postolateral nucleus and the ventral postomedial nucleus. Gustatory information also arrives from the ventral postomedial nucleus via insular cortex (Turner and Herkenham, 1991).

The BLA and BMA receive information from some sensory modalities, including somatosensory and olfactory information, and spacial and contextual input from the hippocampus, project to the BLA and BMA nuclei (Maren, 1999). Olfactory information inputs to both the BLA and BMA from olfactory bulb via piriform cortex while somatosensory information arrives directly to BMA from the medial posterior complex and the parabrachial nucleus. Visual and auditory responses are relayed to the BMA and BLA after entering the LA as part of the intra-amygdaloid circuitry, which will be discussed later.

The central nucleus also receives both ascending and descending extra-amygdaloid input. Descending projections to Ce arrive from cortical areas and thalamic areas. Cortical regions projecting to Ce include viscerosensory and somatosensory regions of insular cortex (infralimbic cortex, dysgranular insular cortex, posterior insular cortex, ventral agranular insular cortex and dorsal agranular insular cortex) (Kapp *et al.*, 1985; Mcdonald *et al.*, 1996; McDonald *et al.*, 1999; Ottersen, 1982; Pascoe & Kapp, 1987; Saper, 1982; Veinante & Freund-Mercier, 1998; Yasui *et al.*, 1991), medial prefrontal cortex (Kapp *et al.*, 1985; Ottersen, 1982), temporal cortex (areas 2 and 3 to CeLC only) (Mascagni *et al.*, 1993), perirhinal cortex (to CeLC only) (McDonald *et al.*, 1999), entorhinal cortex (rostral ventrolateral entorhinal area and rostral dorsolateral entorhinal area) (McDonald & Mascagni, 1997; McDonald *et al.*, 1999), and the amygdalopyriform transition area (McDonald *et al.*, 1999; Shammah-Lagnado & Santiago, 1999). Thalamic regions projecting to Ce include the paraventricular, parataenial, interanteromedial, and parafascicular nuclei, the basal nucleus of the ventromedial complex, the medial division of the medial geniculate complex, posterior intralaminar nucleus and the medial posterior complex (LeDoux *et al.*, 1990; Moga *et al.*, 1995; Ottersen & Ben-Ari, 1979). Ascending projections include hypothalamic input from the ventromedial nucleus, ventral premammilary nucleus, lateral hypothalamus and the posterior hypothalamic nucleus (Canteras *et al.*, 1994; Russchen, 1982; Veening, 1978; Vertes *et al.*, 1995), and brainstem input from dorsal raphe, parabrachial nuclei, locus coeruleus, periaqueductal gray, various tegmental regions, ventrolateral medulla and the nucleus of the solitary tract [(Bernard *et al.*, 1989; Bernard *et al.*, 1993; Hallanger & Wainer, 1988; Halsell, 1992; Jia *et al.*, 1997; Krukoff *et al.*, 1993; Ottersen & Ben-Ari, 1978; Rizvi *et al.*, 1991; Roder & Ciriello, 1993; Russchen, 1982; Veening, 1978; Vertes, 1991).

1.5. INTRAAMYGDALOID PROJECTIONS

Interconnection between the nuclei (internuclear), and indeed the divisions within each nuclei (intranuclear) are highly ordered and quite complex. The passage of information, with a few exceptions (Savander *et al.*, 1997), has largely been generalised to follow a lateral to medial progression from the basolateral amygdaloid complex input station to the central extended amygdala as the output station.

1.5.1. Projections originating in Lateral Amygdala

Axonal tracing studies using tritiated amino acids or the anterograde tracer *Phaseolus vulgaris* leucoagglutin (Pha-L) have demonstrated that neurones of the LA have three main internuclear targets – the BLA, the BMA and the CeL, but they also project heavily within the subdivisions of the LA itself (Krettek & Price, 1978; Pitkänen *et al.*, 1997; Pitkänen *et al.*, 1995; Smith & Paré, 1994).

In the rat (Pitkänen *et al.*, 1995), the lateral division of the LA projects primarily to the BMA, but also projects substantially to the parvicellular division of the BLA, and the amygdalohippocampal area from its caudal end. Light projections were also reported in medial amygdala and the cortical amygdaloid nuclei. The medial division has been reported to project heavily to the BMA, the capsular region of the CeL and the periamygdaloid cortex. Medium density projections are seen in both divisions of the BLA and various corticoamygdaloid areas. The CeL and CeM receive very light LA innervation only, which has now been questioned by the author (A. Pitkänen, 1999, personal communications). A similar pattern of projections was described for the cat (Krettek & Price, 1978; Smith & Paré, 1994) where the laterally situated shell region was found to project primarily to the BMA whereas the medial core region projects to the BLA. In contrast to the study in the rat both regions of LA were found to project to CeL, but as for the rat no projections were identified in the CeM.

Intranuclear projections are heavy and unidirectional between the divisions of the LA (dorsolateral projecting to ventrolateral and medial) but light within these divisions (Pitkänen *et al.*, 1997).

1.5.2. Projections originating in Basolateral Amygdala

The BLA has also been shown to have heavy intranuclear projections, however in contrast to the LA these are mainly confined within the divisions (magnocellular, intermediate and parvicellular). There is light interdivisional projections between the parvicellular division and the other divisions which may also be reciprocal (Pitkänen *et al.*, 1997).

Internuclear projections originating in the BLA primarily target the CeM and nucleus of the lateral olfactory tract (NLOT) and periamygdaloid cortex in the cat, and the amygdalohippocampal area in the rat (Krettek & Price, 1978; Paré *et al.*, 1995; Savander *et al.*, 1997). The BLA also projects back to the LA (Savander *et al.*, 1997), contrary to the generalisation of lateral to medial progression of innervation through the amygdala (Amaral *et al.*, 1992; McDonald, 1992). BLA projections primarily originate in the parvicellular division in the rat, with the only other internuclear projection from the magnocellular division to NLOT (Pitkänen *et al.*, 1997).

1.5.3. Projections originating in the Basomedial Amygdala

Like the BLA, the BMA has extensive inerdivisional projections within its subdivisions (magnocellular and parvicelluar), however unlike the BLA and LA these divisions do not appear to interconnect (Pitkänen *et al.*, 1997).

The internuclear targets of BMA includes the CeM, NLOT, the periamygdaloid cortex, the lateral and anterior cortical nuclei, the central division of medial amygdala, and a reciprocal projection back to LA (Krettek & Price, 1978; Paré *et*

al., 1995; Savander *et al.*, 1997; Savander *et al.*, 1997). These projections arise from both divisions of the BMA (Pitkänen *et al.*, 1997).

1.5.4. Intrinsic connections of the Central Amygdala

The central amygdala has only a few very minor projections to other amygdaloid nuclei, including the BLA, the anterior cortical nucleus the anterior amygdaloid area and the amygdalohippocampal area (Amaral *et al.*, 1992; Jolkkonen & Pitkanen, 1998). It has extensive intra- and interdivisional connections within however, as well as connections to regions included in the CEA – BNST and SI (Grove, 1988; Sun *et al.*, 1991).

The CeLc projects mainly within itself but also to the CeM. Projection to the core region of the lateral division (CeL) is light. The CeL also projects mainly within itself, but also to the CeLc and to the CeM. The CeI and CeM also have heavy intradivisional projections, but in addition the CeM projects to the CeLc (Jolkkonen & Pitkanen, 1998).

1.5.5. Connections with the ICM cells

The intercalated cell masses dispersed in tightly packed islets in the fibre tracts between the main amygdaloid nuclei provide a relay from input cells in the basolateral group to the output group of the Ce (Alheid *et al.*, 1995; Paré & Smith, 1993; Royer *et al.*, 1999). These GABAergic cells have been been shown to receive excitatory inputs from the basolateral nuclei (Royer *et al.*, 1999) and to project into the CeM and CeL (Paré & Smith, 1993; Royer *et al.*, 1999).

1.6. PROJECTIONS FROM THE AMYGDALA

While it now accepted that the central nucleus acts as the primary output station for the amygdala (Pitkänen *et al.*, 1997; Royer *et al.*, 1999) projecting onto brainstem and hypothalamic regions critical for endocrine and autonomic control of homeostasis, the basolateral amygdaloid complex has also been found to project out of the amygdala. These outputs from LA and BLA include reciprocal connections back to corticies (Bacon *et al.*, 1996; Saper, 1982) and the thalamus (Krettek & Price, 1977) and unidirectional projections to substantia innominata, ventral caudate putamen, nucleus accumbens, lateral bed nucleus of the stria terminalis, olfactory tubercle, premammillary nucleus and ventromedial hypothalamic nucleus (Krettek & Price, 1978).

Hypothalamic and brainstem targets of CEA projection neurones include lateral hypothalamus, paraventricular nucleus, dorsal motor nucleus of vagus, nucleus ambiguus, parabrachial nucleus, ventral tegmental area, locus coeruleus, dorsal lateral tegmental nucleus, pontine reticular formation, the central grey, and trigeminal facial motor nucleus (Beitz, 1982; Davis *et al.*, 1994; Jackson & Crossman, 1981; Jongen-Rêlo & Amaral, 1998; Krettek & Price, 1978; Prewitt & Herman, 1998; Rizvi *et al.*, 1991; Schwaber *et al.*, 1980; Schwaber *et al.*, 1982; Takeuchi *et al.*, 1983; Zahm *et al.*, 1999).

1.7. THE CENTRAL EXTENDED AMYGDALA: AN INHIBITORY INTERFACE

As well as sharing and efferent fibre targets in the brainstem and hypothalamus (Beitz, 1982; Jackson & Crossman, 1981; Moga *et al.*, 1989; Schwaber *et al.*, 1980; Schwaber *et al.*, 1982; Weller & Smith, 1982), each CEA region (Ce, IS and BNST) also receives reciprocal connections from these regions (Bernard *et al.*, 1993;

Canteras *et al.*, 1994; Grove, 1988; Halsell, 1992; Krukoff *et al.*, 1993; Ottersen, 1980; Sofroniew, 1983; Swanson *et al.*, 1984; Vertes *et al.*, 1995). The BLA and some cortical areas also provide input to all three regions of CEA (Grove, 1988; Saper, 1982). The projection from the basolateral amygdaloid nuclei to the Ce has been shown to provide excitatory input, as well as feedforward inhibitory input via the ICM (Paré & Smith, 1993; Royer *et al.*, 1999), whereas the primary target of the cortical afferents has also been demonstrated to be the GABAergic interneurones in the Ce (Sun *et al.*, 1994). The primary target of the aforementioned ascending inputs to the CEA is also thought to be these intrinsically projecting GABAergic neurones (Sun *et al.*, 1994). These locally projecting neurones form extensive intrinsic interconnections between the Ce, BNST and SI (Grove, 1988; Sun *et al.*, 1991; Weller & Smith, 1982). This has led to the proposal that an inhibitory interface gates the information flow between cortical and BLA afferents and the brainstem projection neurones in the CEA (Sun *et al.*, 1994).

Under the scheme proposed by Sun *et al* (1994), the projection neurones of the CEA are under constant inhibitory control from activation of local GABAergic connections. On the afferent side, these inputs perform an integrating role for information passing into the CEA (Royer *et al.*, 1999), whereas on the efferent side the reciprocal projections back to the CEA provide a master switch to rapidly disinhibit the projection neurones (through inhibitory inputs onto the GABAergic cells of the inhibitory network) (Sun *et al.*, 1994). This master switch (as noted by Sun *et al* (1994) would be a rapid means for activating short stimulus unconditioned startle responses.

While the concept of the central extended amygdala is now widely accepted (Cassell *et al.*, 1999), the interactions between the Ce, BNST and SI is not well understood. The Ce, BNST and SI have been shown to have similar efferent targets and to receive similar input and each region is able to effect behavioural (eg startle responses (Hitchcock & Davis, 1991; Walker & Davis, 1997)) and autonomic pathways (eg. cardiovascular regulation (Gelsema *et al.*, 1993; Gelsema *et al.*, 1987)). However, the respective roles of each region in mediating these responses is unclear. Recently it has been proposed that regions within the CEA mediate different aspects of behavioural and automonic responses (Davis & Shi, 1999).

Under this proposal the BNST and the Ce may have similar effects on behavioural and autonomic systems but are activated under different afferent circumstances. Evidence supporting this has come from lesioning studies showing that fear potentiated startle can be blocked by lesioning the Ce, whereas light enhanced startle is not. Alternatively, lesions of the BNST can block the light enhanced startle reflex but have little effect on fear potentiated startle (Walker & Davis, 1997). Lesions of the basolateral amygdaloid complex blocked both fear-potentiated and light enhanced startle, indicating that the output each region was dependant on some basolateral input.

1.8. THE SYNAPTIC PHYSIOLOGY OF THE CENTRAL AMYGDALA

In contrast to other regions of the brain (hippocampus, cortex), the circuitry of the amygdala has not been well established. Despite the work of numerous anatomists showing afferent, intrinsic and efferent connectional data, histochemical and cytoarchitectonic studies of cellular morphology, the nature of the synaptic transmission between the limbic structures has yet to be fully explored. The amygdala is often divided into a cortical-like input side and a striatum-like output side. To date, electrophyisological studies (with only eight exceptions) have examined the physiology of synaptic connections in the cortical-like areas of the basolateral complex and the associated cortices. Some understanding of the synaptic physiology of these regions, have led to the proposals that this area may be the site of some plasticity involved in the behavioural functions of the amygdala (Faneslow & LeDoux, 1999; Rogan & LeDoux, 1996).

The emergent picture of the central extended amygdala is also of a site capable of some processing involved with behavioural function. The innervation of this region by cortical afferents, and potential of the intrinsic GABAergic inhibitory network to modulate the ouput of this structure suggest that it too may perform an integrative

role in amygdaloid function (Royer *et al.*, 1999; Sun & Cassell, 1993; Sun *et al.*, 1991; Sun *et al.*, 1994). The few studies which have examined the synaptic inputs onto central amygdaloid cells have until recently (Martina *et al.*, 1999; Royer *et al.*, 1999), been done using sharp electrode techniques and have not discriminated between the subdivisions of the Ce despite reported connectional and cytoarchitectonic differences between these regions. The work contained in this thesis attempts to characterise the synaptic currents underlying the EPSP and IPSP in Central Amygdala, using established pharmacological criteria. To do so we have employed the blind whole cell patch clamp technique and recorded from neurones held in voltage clamp mode. The research in this thesis and that being performed in Denis Paré's laboratory in Canada, are the first attempts at whole cell recording from neurones of the central amygdala.

2. METHODS

2.1. PREPARATION OF BRAIN SLICES

Wistar rats 17-28 days old were anaesthetised with intraperitoneal pentobarbitone (50 mg/Kg body weight) and decapitated just prior to death according to institutional animal ethics committee guidelines.

The scull was exposed, cut along the midline, and removed using 14 mm rongeurs. The exposed brain was then removed, blocked coronally and glued onto the vibratome or microslicer cutting block and immersed in ice cold ACSF. The brain was then hemisectioned, and coronal slices (400 μm thick) cut through the entire rostrocaudal extent. Slices were removed immediately, transferred to an oxygenated slice chamber containing ACSF at 30°C, and allowed to recover for 30 mins prior to recording. Slices were maintained at room temperature in oxygenated ACSF (In mM: NaCl 118, KCl 2.5, $NaHCO_3$ 25, glucose 10, NaH_2PO_4 1.2, $MgCl_2$ 1.3, $CaCl_2$ 2.5) for several hours.

2.2. RECORDING CONDITIONS

Slices were placed in a perspex perfusion chamber of with bath volume of 1 ml, retained under a nylon stranded net, and superfused at 200 ml per hour with oxygenated ACSF at room temperature. Slices were visualised using a stereo dissection microscope (ISSCO) under translumination.

Whole cell recordings were made using 3-5 MΩ borosilicate glass electrodes using the blind approach (Blanton *et al.*, 1989). Internal solutions used included potassium methyl sulfate ($KmeSO_4$) internal (in mM: $KmeSO_4$ 135, NaCl 8, HEPES 10,

MgATP 2, NaGTP 0.3, EGTA 0.2, osmolarity :280-290 mOsM, pH 7.3 with KOH), cesium chloride (CsCl) internal (in mM: CsCl 130, $MgCl_2.6H_2O$ 1, EGTA 10, HEPES 10, MgATP 2, NaGTP 0.2, osmolarity 280-290 mOsM, pH 7.3 with CsOH) and cesium gluconate (CsGluc) internal (in mM: gluconate 107.5, CsCl 17.5, NaCl 8, HEPES 10, MgATP 2, NaGTP 0.2, BAPTA 10, osmolarity: 280-290 mOsM, pH 7.3 with CsOH). Membrane potentials recorded using the Cs Gluc internal were corrected for a junction potential of +17 mV. Recordings were made in both current clamp and voltage clamp configurations, and series resistance (10 - 30 MΩ) monitored throughout the experiment. No series resistance compensation was used and experiments were rejected if series resistance changed by more than 10%.

Synaptic responses were evoked electrically using stainless steel bipolar stimulating electrodes (Frederick Haer) connected to Digitimer model DS2A stimulator boxes. Stimuli were 1-40 V in amplitude and 50 μs in duration.

Iontophoretic applications were made using borosilicate glass pipettes (>5 MΩ) filled with acidic solutions. Negative retention currents (50-100 nA) and positive ejection current (100-200 nA, 0.1-1 s duration) were generated by a Dagan 6400 iontophoresis unit. GABA and glycine solutions used were 300 mM (adjusted to pH 3 with HCl).

Hypertonic sucrose (500 mM in ACSF) was applied by pressure injection through a borosilicate glass pipette (3-5 MΩ). Blind application was made by sustained pressure injection with the pipette placed on the surface of the slice. Localised applications using IR/DIC techniques were made by brief low pressure applications.

2.3. DATA COLLECTION, ANALYSIS AND STATISTICS

Signals were filtered (5 kHz) and amplified using either an Axopatch 1D or an Axopatch 200B amplifier (Axon instruments), digitised at 10 kHz using an ITC-16

analogue to digital converter (Instrutech), and recorded on an Apple Macintosh computer (PowerMac or G3) or onto video tape using a Vetter PCM recorder (model 200, A.C. Vetter Co.). Computer data acquisition was facilitated by Igor Pro software or by Axograph 4.0 software (Axon instruments). All data analysis was performed using Axograph 4.0 software. Results were compiled and analysed using Microsoft Excel. All values shown are mean ± SEM, and statistical comparisons were done using student's *t* test.

2.4. DRUGS USED

Kynurenic acid (Sigma, RBI research chemicals); used at 2 mM, (1 M stock).

CNQX (Tocris Cookson); used at 10 μM, (10 mM stock).

D-APV (RBI research chemicals); used at 30 μM, (30 mM stock).

Bicuculline methiodide (Sigma, RBI research chemicals); used at 10 μM (except where otherwise stated), (10 mM stock).

Picrotoxin (Sigma); used at 100 μM (except where otherwise stated), (100 mM stock).

SR95531 (Sigma); used at 2 μM (except where otherwise stated), (1 mM stock).

TPMPA (RBI research chemicals); used at 60 - 100 μM (except where otherwise stated), (100 mM stock).

Strychnine (Sigma); used at 1 μM (except where otherwise stated), (10 mM stock).

Tetrodotoxin (Alamone); used at 0.5 μM, (1 mM stock).

Propofol (RBI research chemicals); used at 10 μM, (10 mM stock).

Pentobarbitone (Bomac Laboratories); used at 25 μM, (25 mM stock).

Diazepam (gift from Professor P. Gage) ;used at 1 or 10 μM, (10 mM stock).

Flurazepam (gift from Professor G.A.R. Johnston); used at 1 μM, (1 mM stock).

Ro 15-1788 (gift from Professor G.A.R. Johnston); used at 1 μM, (1 mM stock).

3. MEMBRANE PROPERTIES AND SYNAPTIC CURRENTS OF Ce NEURONES

3.1. INTRODUCTION

Of seven publications showing intracellular electrophysiological recordings made from central amygdala neurones, three have recorded in rat brain slices (Nose *et al.*, 1991; Rainnie *et al.*, 1992; Schiess *et al.*, 1993), three in guinea pig brain slices (Martina *et al.*, 1999; Nose *et al.*, 1991; Royer *et al.*, 1999) and two in dissociated neurones (Yu & Shinnick-Gallagher, 1998; Yu & Shinnick-Gallagher, 1997).

The first of these papers by Nose *et al* examined electrically evoked post synaptic potentials (PSPs) in the whole of the Ce nucleus (and the results shown are all recordings from guinea pig). This paper reported excitatory PSPs (EPSPs) elicited by stimulating in the BLA and inhibitory PSPs (IPSPs) when stimulating in the dorsal region of the lateral division of the Ce. The EPSPs were shown to be mediated by the AMPA and NMDA glutamate receptors, and by nicotinic receptors sensitive to d-tubocurarine and hexamethonium. The IPSC was blocked by the $GABA_A$ antagonist bicuculline and the glycine receptor antagonist strychnine, suggesting both GABAergic and glycinergic inhibition.

Rainnie *et al* examined the effects of corticotrophin releasing factor on Ce neurones in a paper published in 1992 (Rainnie *et al.*, 1992). This paper showed some passive membrane properties, the afterhyperpolarisation (AHP) and a hyperpolarising effect of corticotrophin releasing hormone on some Ce neurones which reduced the slow AHP in these neurones. The same group published another paper in 1993 (Schiess *et al.*, 1993) that was concerned with the firing properties and AHPs of Ce neurones. This paper sought to classify the Ce neurones according to these properties.

Other than the most recent papers (Martina *et al.*, 1999; Royer *et al.*, 1999) previous studies have examined the properties of the neurones of the whole Ce nucleus, without reporting or discussing subdivisional differences. As these recent papers, and complimentary anatomical studies have shown, the divisions of the Ce represent distinct regions of connectivity (Pitkänen *et al.*, 1997) and include neurones of distinct cytological properties (McDonald, 1982).

In contrast to the earlier Sheiss *et al* study (Schiess *et al.*, 1993), Martina *et al* reported nine distinct neuronal subtypes in the lateral and medial divisions of Ce based on membrane properties and firing patterns (Martina *et al.*, 1999). This study also described the morphology of CeL and CeM neurones. Notably, differences were observed both between the cells of each division, although different classes of cells within the CeL appeared to have similar cellular morphology. The same group more recently published a study examining the synaptic responses of neurones in CeL and CeM (Royer *et al.*, 1999). This study indicated that CeL neurones responded to electrical stimulation in the LA and BLA, whereas CeM neurones responded to BLA and BMA stimulation. This paper also showed that IPSPs recorded in CeM neurones in response to stimulation of the nuclei of the basolateral complex nuclei, arrive via a feed forward inhibitory pathway involving a network of interconnected ICM cells located between the basolateral nuclei and the central nucleus. These papers suggest a far more complex scenario for the circuitry and cellular properties of the Ce than reported previously by Scheiss *et al* and Nose *et al.*

This chapter shows the first whole cell recordings made from Ce neurones in rat. The objective of this chapter is to examine membrane and firing properties of rat Ce neurones and the synaptic currents of the neurones generated by stimulation within the basolateral complex nuclei.

3.2. RESULTS

3.2.1. Recordings from CeL neurones

CeL neurones were held in current clamp mode at Vm –60 (using potassium methyl sulfate ($KmeSO_4$) based internal solution) and the responses to 600 ms current injections from –300 pA to 500 pA, recorded. Six neurones were held in current clamp and a series of increasing current steps from –300 pA to +400 pA were recorded. Three neurones showed firing patterns featuring delayed firing (Figure 3.1.A), two fired just one or two spikes rapidly and then stopped firing completely (Figure 3.1.B), and one neurone fired repetitively over the entire length of the current step (Figure 3.1.C).

All three firing patterns recorded, correspond to neurone types seen in guinea pig by Martina *et al* (Martina *et al.*, 1999). The first group of neurones (delayed firing neurones) appear similar to the late firing neurones reported in guinea pig CeL and were similarly the most frequently encountered. The rapidly spiking neurones which fired single spikes probably correspond to the fast-spiking neurones of the guinea pig CeL, although the CeL neurones do not fire repeatedly like the guinea pig cells. Repetitive firing neurones were also encountered in the guinea pig CeL.

3.2.2. Recordings from CeM neurones

Using a similar protocol as used previously for CeL neurones, the firing patterns of CeM neurones could be classified into two classes. The most prominent class (6 of 9 CeM neurones recorded) were delayed firing neurones (Figure 3.2.A). The remaining neurones were all rapid firing neurones which displayed a characteristic spike on the off phase of the current injection (Figure 3.2.B).

As for the CeL, the neurones recorded in CeM corresponded to classes of neurones reported by Martina *et al* in the guinea pig (Martina *et al.*, 1995). Late firing neurones were the most prominent firing class in both species and both had a class

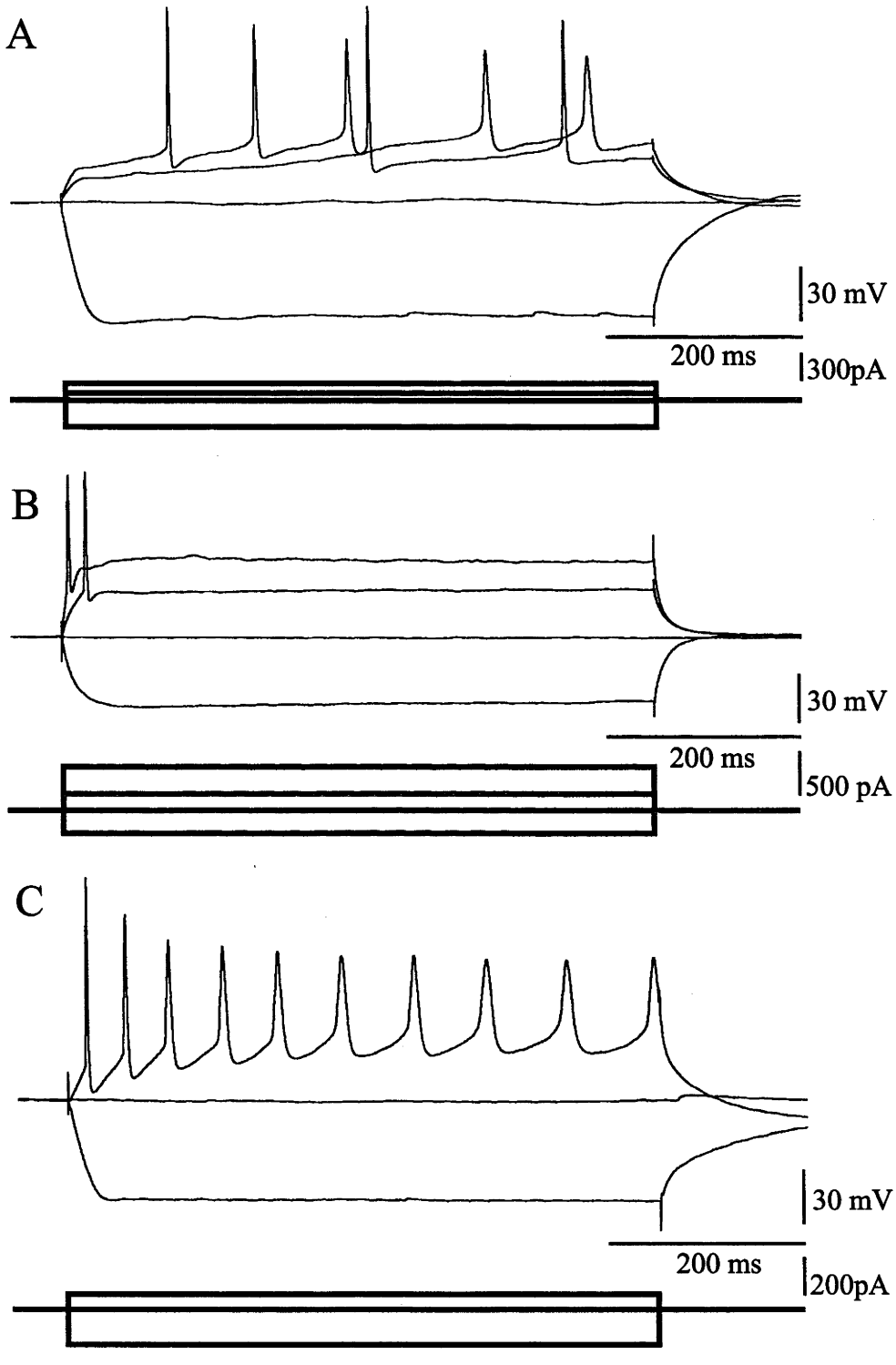

Figure 3.1. Firing patterns of CeL neurones in response to current injections.

A. Delayed firing neurone, B. single firing neurone and C. repetitive firing neurone. Neurones were held at Vm -60 mV, and responses to increasing current injections (-300 pA to 500 pA in 50 pA increments) were recorded.

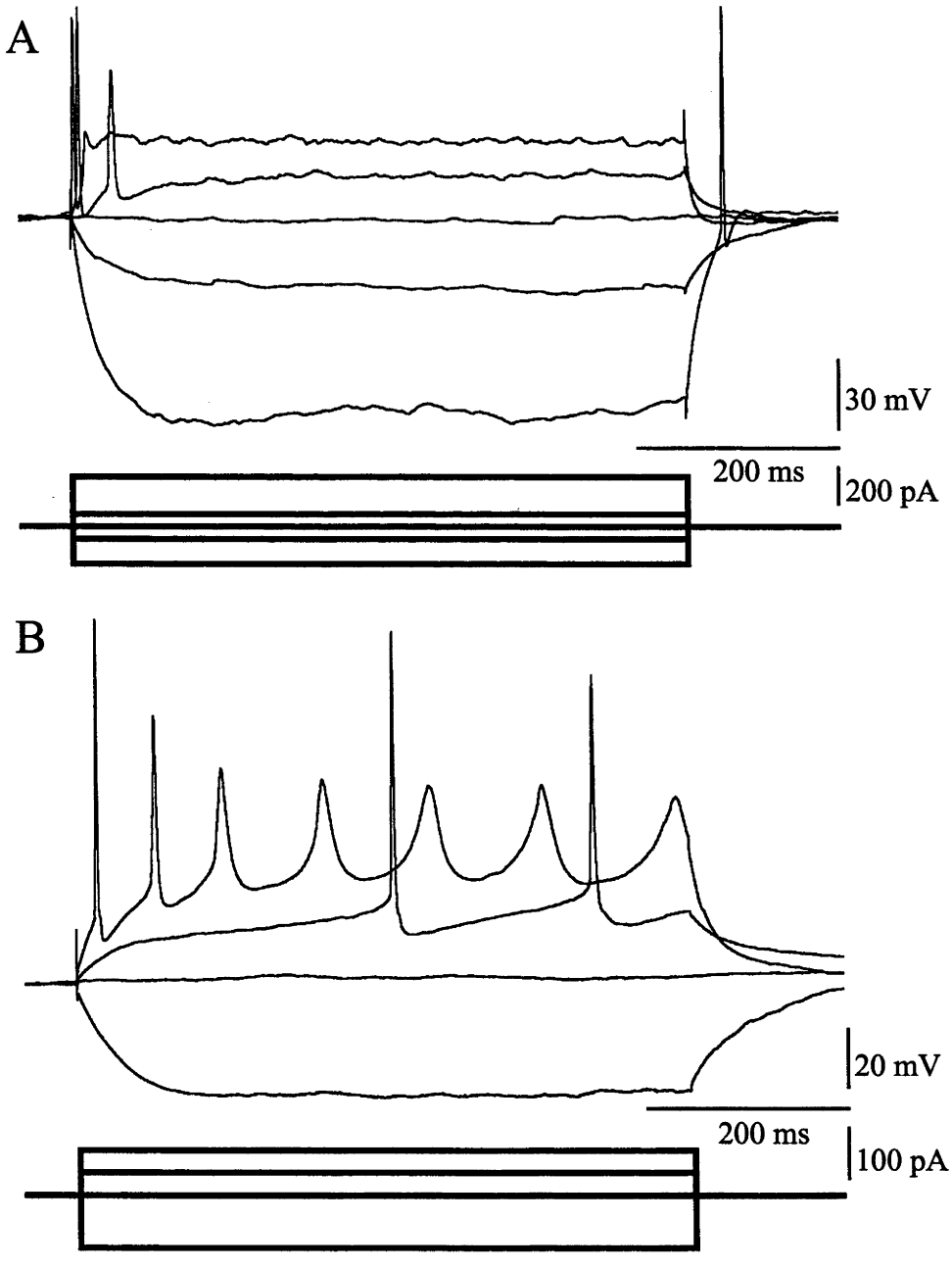

Figure 3.2. Firing patterns of CeM neurones in response to current injections.
A. Rapidly accomodating neurone (with spike on the off phase of the current injection), and B. late firing neurone. Neurones were held at Vm -60 mV, and response to increasing current injections (from -200 pA to 300 pA in 50 pA increments) were recorded.

of fast spiking neurones. The rat fast spiking cells again showed only single spikes, whereas the guinea pig fast spiking cells had spiked repeatedly.

3.2.3. Synaptic responses of Ce neurones

Recording in voltage clamp mode, synaptic responses to electrical stimulation of the three basolateral nuclei were recorded from 43 CeL and 19 CeM neurones, using $KmeSO_4$ or cesium gluconate (CsGluc) based internal solutions. The chloride reversal potential for these internal solutions (-60 and –50 mV respectively) allowed us to record at subthreshold potentials (Vm -40), the inhibitory post synaptic current (IPSC) mediated by the fast ionotropic GABA or glycine receptors, as an outward current (an influx of anionic chloride). The excitatory post synaptic current response (EPSC) appeared as inward currents (cationic sodium and potassium influx) at the reversal potential for chloride.

Synaptic responses were recorded for electrical stimulation of LA, BLA, and the dorsal region of CeL itself (n = 25, 26 and 14 respectively). Control recordings from these regions produced an inward/outward current pair (EPSC/IPSC) at Vm -40 and inward currents (EPSC) at the chloride reversal potential (Figure 3.3.).

Stimulation of the BLA and LA produced no synaptic response in CeM neurones in contrast (n= 6 and 5 respectively) but synaptic responses were elicited by stimulating the BMA (n = 10) and the CeL (n = 15) (Figure 3.4.). These responses also consisted of EPSC/IPSC responses recording at membrane potential of -40 mV, and an EPSC only at the chloride equilibrium potential.

3.2.4. Inhibitory inputs from LA and BLA to CeL are disynaptic

The IPSC recorded in CeL neurones in response to stimulation in the LA and BLA was blocked by the application of the glutamate receptor blocker kynurenic acid (2 mM) (n = 4, Figure 3.5.A). This indicates that the IPSC recorded in CeL stimulating these regions results from the firing of a second neurone between the LA and BLA,

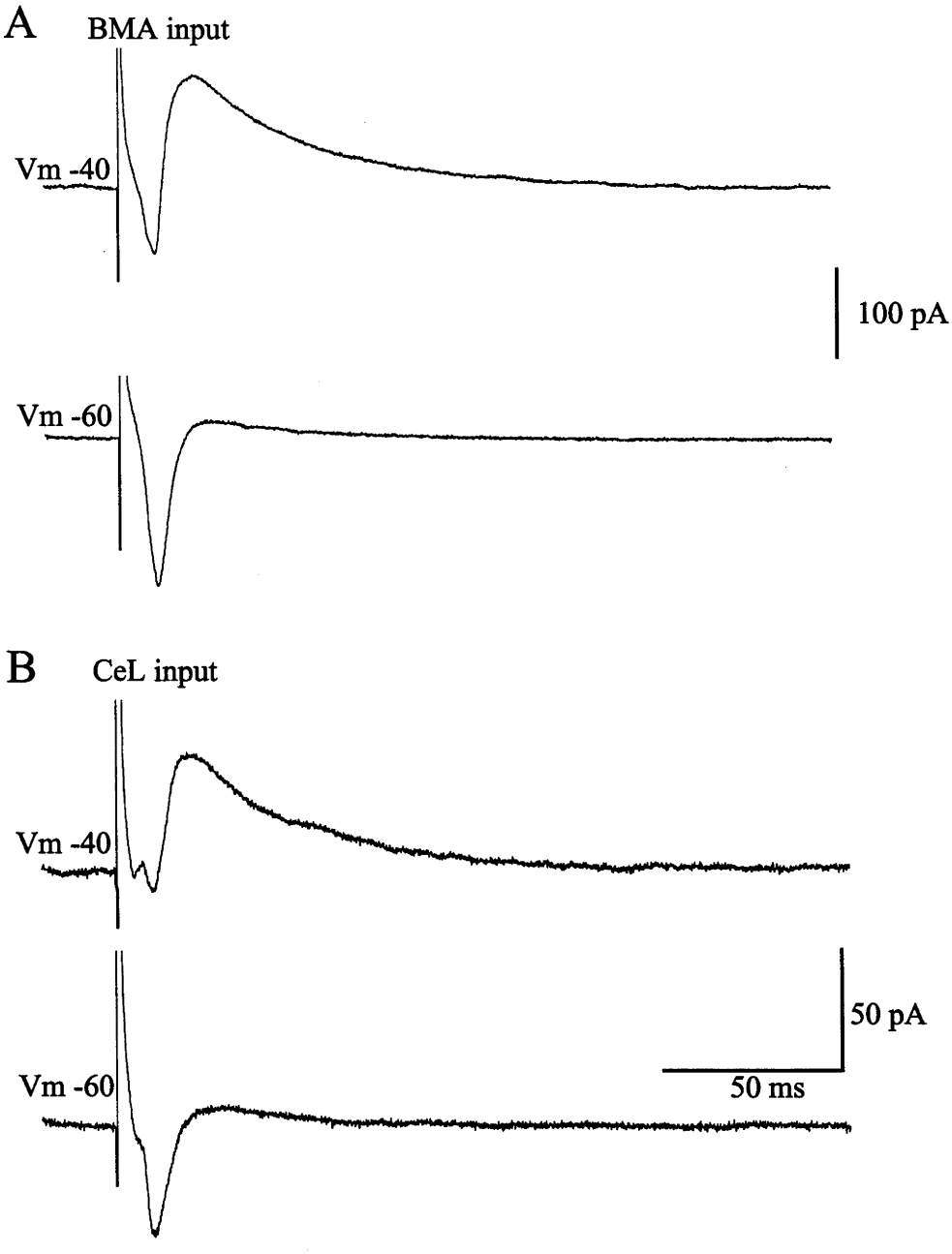

Figure 3.3. Synaptic responses in CeM neurones.
Averages of 10 synaptic responses recorded at Vm -40 and -60 when stimulating electrically in BMA. (A) and CeL (B). The internal solution used was $KmeSO_4$ based, with reversal potential for chloride at Vm -60 mV.

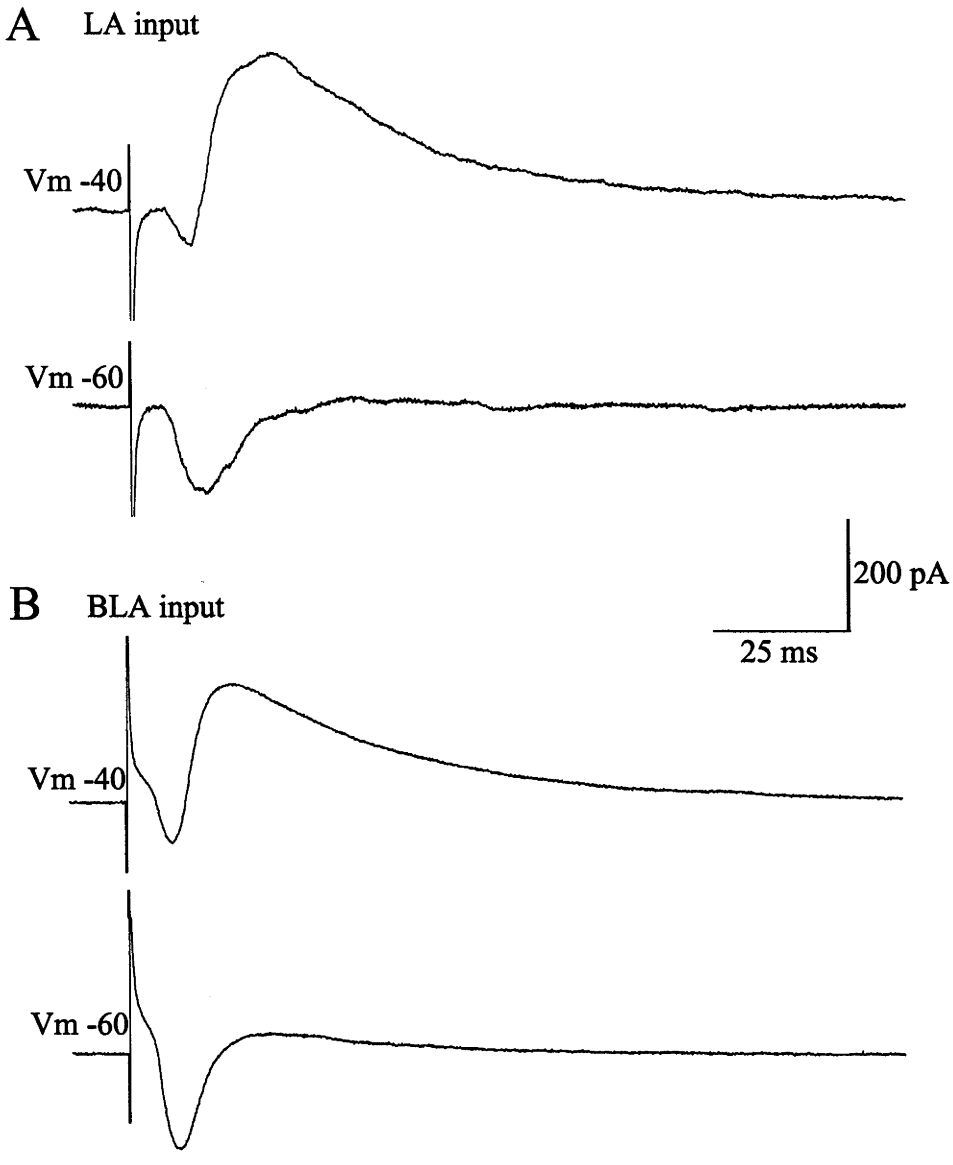

Figure 3.4. Synaptic responses in CeL neurones.
Averages of 6 synaptic responses recorded at Vm -40 and -60 when stimulating electrically in LA. (A) and BLA (B). The internal solution used was $KmeSO_4$ based, with the reversal potential for chloride at Vm -60 mV.

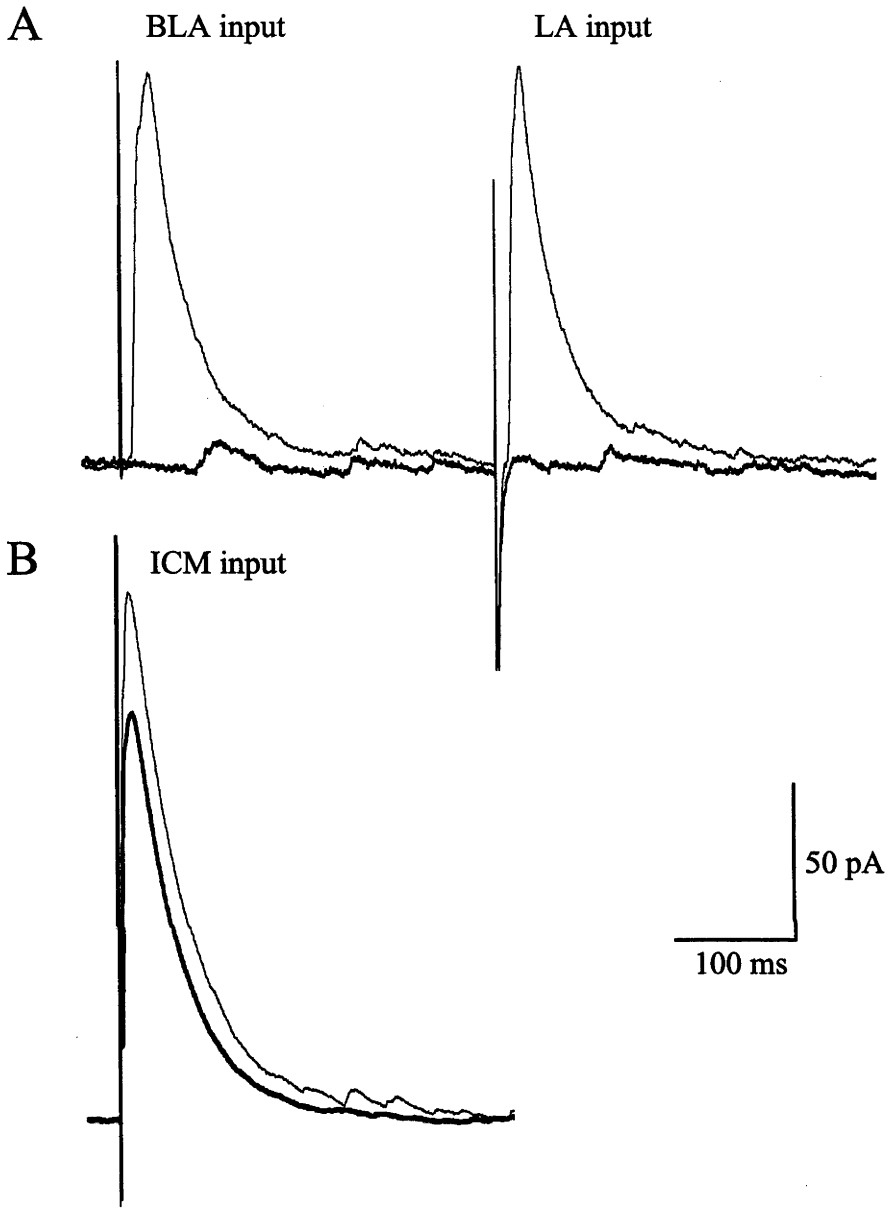

Figure 3.5. Disynaptic and monosynaptic inhibitory inputs to CeL neurones.
A. IPSC evoked by electrical stimulation in the BLA and LA in control and blocked (heavy line) after application of kynurenic acid (2 mM). B. IPSC evoked by stimulation in the ICM area between LA and Ce, in control (light line) and in kynurenic acid (heavy line). All recordings were made at Vm 0 using cesium gluconate based internal solution (corrected for -17 mV junction potential).

and the CeL. Moving the stimulators onto the edge of the CeL – in the vicinity of the ICM cells in the internal capsule, an IPSC was elicited which was not blocked by kynurenic acid (Figure 3.5.B). Similarly, the IPSC recorded when stimulating in the CeL itself was not blocked by kynurenic acid.

An IPSC was recorded in CeM in the presence of kynurenic acid when stimulating in CeL and the BMA (n = 6 and 4 respectively), indicating direct GABAergic projections.

3.2.5. Excitatory Currents of Ce neurones

The Ce EPSC could be isolated in the presence of the GABA/Glycine receptor antagonist picrotoxin. Recording in either CeL or CeM using CsGluc based internal, the EPSC was a fast rising and decaying inward current at negative membrane potentials, and a fast rising and slow decaying outward current at positive membrane potentials. Recording these responses at a range of membrane potentials from –100 mV to +40 mV (Figure 3.6.A), the amplitude of the response at the peak of the inward current plotted against the membrane potential resulted in a linear I/V relationship passing through Vm 0 (Figure 4.6.B open). This voltage insensitive I/V relationship and the fast kinetics of the inward current recorded at hyperpolarised membrane potential is typical of excitatory currents mediated by AMPA type glutamate receptors (Hestrin *et al.*, 1990). The amplitude of the slow decaying outward current (measured at 140 ms post stimulus) similarly plotted against membrane potential (Figure 3.6.B filled), resulted in a voltage dependant I/V relationship typical of NMDA receptor mediated currents, with a region of negative slope between membrane potentials -70 and –30 mV (Hestrin *et al.*, 1990). Confirming this, the slow decaying component was blocked by the specific NMDA antagonist D-APV (30 μM) leaving just the fast rising and decaying component (Figure 3.6.C). This component was blocked by the specific AMPA receptor antagonist CNQX (10 μM) (Figure 3.6.D), confirming that this component of the EPSC was an AMPA receptor mediated current.

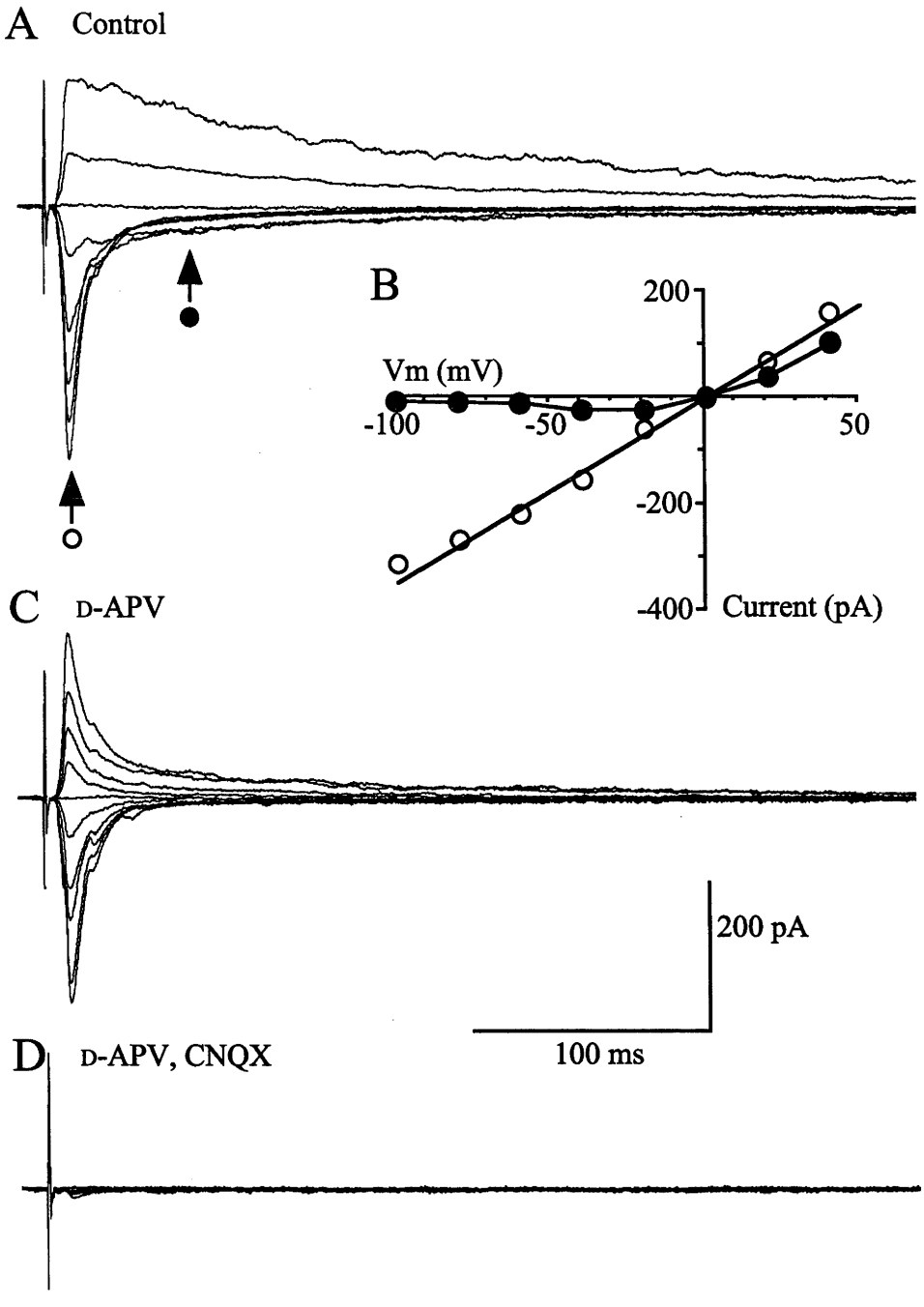

Figure 3.6. Excitatory currents in Ce neurones are mediated by AMPA and NMDA receptors.

A. Control recordings of EPSCs (in 100 µM picrotoxin) at membrane potential -97 to +43 mV* using cesium gluconate based internal. B. The amplitude of these responses at 10 ms and 140 ms post stimulus (indicated by open and closed circles respectively) plotted against the membrane potential. C. EPSCs recorded at membrane potential -97 to +43*, in the presence of extracellular D-APV (30 µM) and D. after addition of CNQX (10 µM). *Membrane potentials corrected for -17 mV junction potential.

3.2.6. Monosynaptic IPSC are chloride currents

Recording in the presence of 2 mM kynurenic acid, a monosynaptic IPSC was elicited by stimulating in the ICM region immediately lateral to the CeL. This IPSC was recorded at membrane potentials from –100 mV to 0 mV using CsGluc based internal solution (Figure 3.7.A), and –80 to +40 mV using cesium chloride internal (CsCl). These currents reversed at the chloride equilibrium potential for either internal solutions (-52 mV and 0 mV respectively) as shown on the average normalised I/V relationship (Figure 3.7.B) (n = 4 for CsGluc, n = 2 for CsCl).

Application of the $GABA_A$ antagonist bicuculline to the external solution resulted in a reduction of the IPSC amplitude, indicating that $GABA_A$ receptors were contributing to the IPSC. The Residual IPSC was also found to reverse at the chloride reversal potential for the CsCl and CsGluc internals used (Figure 3.7.C,D), indicating that this IPSC was also mediated by a chloride conductance.

3.3. DISCUSSION

Although the sample of neurones recorded was small, the proportion of cell types of similar firing types as those previously shown in guinea pig was consistent with the proportions of these types in that species [Martina, 1999 #2048]. Biocytin labeling of the Ce neurones was not successful, so no correlation of these firing properties could be made with cell morphology. Filling neurones with lucifer yellow was also unsuccessful as no filled cells were seen in the slice when examined under UV light immediately after removal of the recording pipette. It is likely that the cells recorded from were removed from the slice on withdrawal of the recording pipette, possibly as a result of the small size of Ce neurones and the recording conditions.

The firing patterns exhibited by the majority of Ce were either rapidly accomodating (firing a single spike at the onset of the current injection) or delayed firing (firing at the middle). These firing patterns are most likely influenced or a result of the

presence of voltage gated potassium channels on the soma or dendrites of the cells, and the activation and deactivation kinetics of these channels. It is possible that recording from soma verses dendritic locations may influence the firing pattern in response to a current injection if for example the potassium channels are differentially localised to soma or dendrites and were more or less activated in response to the current injection at one point verses the other. However, it was unlikely that recordings were made from dendrites locales as these experiments were carried out using recording pipettes of approximately 3Mohm resistance and the dendrites of these cells are very thin. Thus the most likely explanation for the different firing patterns found is a distribution of cell types in each location or different voltage gated potassium channel phenotypes.

The synaptic responsiveness to stimulation in the various basolateral nuclei was also similar to that shown for guinea pig [Royer, 1999 #1964], with the exception that in this study no synaptic responses were elicited in CeM when stimulating in the BLA. The CeL was found to receive excitatory and disynaptic inhibitory input from stimulation in the LA and BLA, whereas the CeM received direct inhibitory and excitatory input from stimulation in the BMA region and the CeL.

The use of the bipolar recording stimulators as in this study does not distinguish between inputs that are situated in the area of stimulation and axons that pass through this region (fibres of passage). For instance, while axonal tracing studies have shown that LA does project to the CeL [Smith, 1994 #1922], fibres from the cortical input to the CeL may also pass through the LA region (A Pitkannen personal communications,1999). Thus though it is likely that the EPSC in CeL stimulated in LA results from activation of LA neurones it is possible that cortical afferents passing through the LA region stimulated may contribute to the responses recorded. Furthermore, the disynaptic IPSC recorded in the CeL when stimulating in the LA may also result from cortical or LA innervation of these GABAergic inputs. Also, despite the fact that the proximity of the stimulation site for eliciting the momosynaptic IPSC in the CeL is suggestive of excitation of neurones in the ICM or CeL itself, and these regions have both been shown to innervate Ce neurones [Jolkkonen, 1998 #1965; Royer, 1999 #1964], there is also the possibility that another nucleus provides this inhibition and the axons from this region pass through

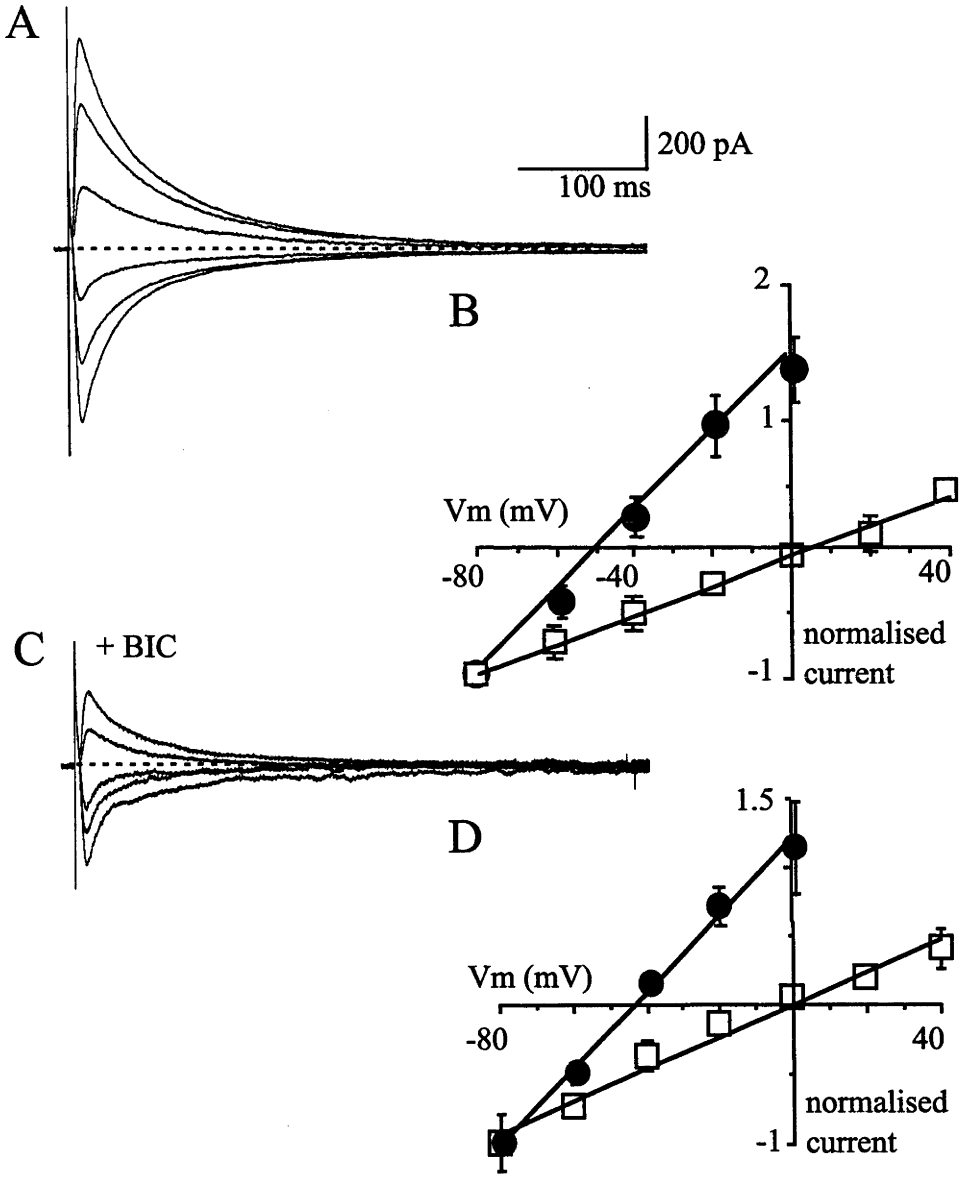

Figure 3.7. Monosynaptic inhibitory currents in CeL are chloride currents. A. IPSCs recorded in a CeL neurone in the presence of kynurenic acid (2 mM), at membrane potentials of -97, -77, -57, -37, -17 and +3 using cesium gluconate internal solution. B. Normalised I/V relationship for IPSCs recorded using cesium gluconate internal (filled circles) and cesium chloride internal (open squares). Reversal potentials were -52 and 0 mV respectively. C. IPSC in the presence of bicuculline (10 μM), recorded at Vm -97 to +3 mV, using cesium gluconate internal solution. D. average normalised I/V relationship for similar IPSC recorded using cesium gluconate (filled circles) and cesium chloride (open squares) internal solutions. Reversal potentials were -44 and 0 mV respectively. Membrane potentials have been corrected for -17 and 10 mV junction potentials for cesium gluconate and cesium chloride internals respectively.

this ICM region. While these results do not resolve the complex circuitry projecting to the Ce, they do reflect the possibilities for innervation provided by tracing studies of the extrinsic connections of the Ce.

The pharmacology of the excitatory and inhibitory currents recorded in CeL and CeM showed some similarity to that previously reported. Nose *et al* identified the receptors underlying the EPSP as the NMDA and non-NMDA type glutamate receptors by blocking with the respective antagonists D-APV and CNQX [Nose, 1991 #1996]. The same group also described an EPSP that was sensitive to d-tubocurarie and hexamethonium, and an IPSP not blocked by bicuculline but blocked by strychnine. The current underlying the putative nicotinic EPSP was not found in neurones when recording with $KmeSO_4$ internal, however the block of the IPSC by bicuculline (10 μM) was not complete, confirming the presence of an additional inhibitory mechanism.

4. INHIBITORY SYNAPSES ON CE NEURONES

In the previous chapter, the inhibitory post synaptic currents of CeL neurones were found to be chloride currents mediated by receptors partially blocked by low concentrations of bicuculline. This chapter investigates the type of ionotropic receptor mediating the IPSC in CeL neurones and the pharmacology of these receptors.

4.1. GABA

The debate as to whether nervous transmission was chemical or electrical had raged since the 1930's, but as new physiological techniques using micropipettes became available in the 1950's, transmission had been shown to be chemical (Brock *et al.*, 1952) and the mechanisms underlying the EPSP's and IPSP's were beginning to be understood (Coombs *et al.*, 1955; Coombs *et al.*, 1955; Coombs *et al.*, 1955; Eccles, 1964). Research by Paul Fatt and Bernard Katz in the early 50s had established acetylcholine as a putative chemical transmitter mediating nerve to muscle transmission, however the nature of the chemical transmitters in other regions had not been established. In 1956 Bazemore *et al* identified the amino acid γ-aminobutyric acid (GABA) as the principle component of the inhibitory chemical transmitter which had been called Factor I (Bazemore *et al.*, 1956). A number of reports then appeared in rapid succession from 1957 to 1959 showing depressant effects of the amino acid γ-aminobutyric acid (GABA) in various nervous tissue preparations (Curtis *et al.*, 1959; Edwards & Kuffler, 1959; Edwards & Kuffler, 1957; Eidelberg & Feldman, 1958; Enger & Burgen, 1957; Iwama & Jasper, 1957; Marrazi *et al.*, 1958; Purpura *et al.*, 1957; Sigg & Grundfest, 1958). David Curtis, working in laboratories in this institute, was examining the effects of what he termed excitant and depressant amino acids on isolated spinal cords. This research demonstrated that both GABA and glycine produced neuronal membrane

hyperpolarisations consistent with those described in the analysis of the IPSC by Eccles *et al* (Coombs *et al.*, 1955; Curtis *et al.*, 1961). GABA was subsequently shown to be localised to mammalian nerve terminals (Neal & Iversen, 1969), from where it is released in a calcium-dependant manner in response to depolarising stimuli (Bradford, 1970).

GABA is now accepted as the major inhibitory transmitter in the mammalian CNS (Nicoll *et al.*, 1989). Its actions can be divided into fast ionotropic receptor mediated inhibition which produces a rapid and acute inhibitory hyperpolarisation (Costa, 1998; Mody *et al.*, 1994), prolonged metabotropic inhibitory hyperpolarisation which may modulate spontaneous activity (Mott & Lewis, 1994), and presynaptic metabotropic inhibition of release (Ault & Nadler, 1982; Blaxter & Carlen, 1985; Lanthorn & Cotman, 1981).

4.2. GABA RECEPTORS

GABA receptors have been classified into ionotropic types ($GABA_A$ receptors and $GABA_C$ receptors) and a metabotropic type ($GABA_B$). The ionotropic receptors gate chloride channels formed through the structure of the receptor complex. The metabotropic $GABA_B$ receptor is linked via transduction proteins called G proteins to effector potassium ion channels and other intracellular processes (Mott & Lewis, 1994).

4.2.1. $GABA_A$ Receptors

$GABA_A$ receptors were originally designated as those GABA receptors which are GABA-gated chloride channels blocked by the competitive antagonist bicuculline (Hill & Bowery, 1981). They have been localised to the synaptic specialisation and extra-synaptically (Nusser *et al.*, 1998), and both pre- and post-synaptically (Johnston, 1996).

Structurally, $GABA_A$ receptors are thought to be pentameric combinations of $GABA_A$ subunits (by analogy with the nicotinic receptor - (Unwin, 1989)), and recent electron microscopy appears to support this (Nayeem *et al.*, 1994). Since the first reported cloning of $GABA_A$ subunits ($\alpha 1$ and $\beta 1$ by Schofield *et al* (Schofield *et al.*, 1987), the $GABA_A$ subunit family has grown to include 19 related subunits (table 5.1.). This number is further increased by alternate splicing in at least 5 of these subunits (Barnard *et al.*, 1998). It is thought that these subunits do not randomly combine to form pentamers however. Studies to date indicate that the $GABA_A$ receptors of the CNS are combinations of α and β subunits with one or more γ, δ or ε subunits. The π subunit has only been detected in peripheral tissue, though is has been demonstrated to be able to form functional GABA receptors with α and β subunits in expression systems (Hedblom & Kirkness, 1997).

The consequence of such a large number of subunit combinations is a heterogenic population of GABA receptors with similarly heterogenic channel properties and pharmacological properties. Furthermore, the presence of several subunit combinations forming different functional $GABA_A$ receptors within the same cell and at specific synapses within that cell (Nusser *et al.*, 1996; Nusser *et al.*, 1998) adds yet another level of complexity to $GABA_A$ mediated inhibition.

4.2.1. $GABA_B$ receptors

The lipophilic GABA analogue baclofen was found to activate a class of GABA receptors which was insensitive to bicuculline (Curtis *et al.*, 1974). These receptors were described as $GABA_B$ receptors by Hill and Bowery (1981) who found that these receptors (activated by both GABA and baclofen) depressed noradrenaline release from autonomic nerve terminals in a bicuculline insensitive manner.

Postsynaptic $GABA_B$ mediated inhibition has been shown to be mediated by potassium conductances, which are recorded as slow IPSCs under whole cell voltage clamp. Rise times of these currents are in the order of tens of milliseconds and decay

times in the order of hundreds of milliseconds (Ling & Bernardo, 1994; Ulrich & Huguenard, 1996). The resultant slow inhibitory potentials mediated by $GABA_B$ receptors is prolonged and may last for several seconds (Danober & Pape, 1998; Lambert & Wilson, 1994). Postsynaptic $GABA_B$ activation has also been shown to effect longer term changes in the post synaptic cell through negative regulation of transcription (Barthel *et al.*, 1996), or modulation of other intracellular processes such as the synthesis of IP_3 (Mott & Lewis, 1994).

Presynaptic $GABA_B$ inhibition has been demonstrated to occur at both excitatory (Ault & Nadler, 1982; Blaxter & Carlen, 1985; Lanthorn & Cotman, 1981) and inhibitory terminals (Davies *et al.*, 1990; Misgeld *et al.*, 1989). The mechanism of presynaptic $GABA_B$ inhibition has been the subject of some debate. The two potential mechanisms proposed are; 1) increasing potassium conductance which shunts the action potential at the terminal resulting in less activation of voltage sensitive calcium channels and hence less release and 2) direct negative modulation of the calcium channel via a G-protein linked to the $GABA_B$ receptor with the same ultimate effect (Mott & Lewis, 1994). Initially the potassium conductance mechanism was discounted as the presynaptic action of the specific $GABA_B$ agonist baclofen was not blocked by extracellular barium. Recently however, postsynaptic $GABA_B$ responses have been shown in the presence of extracellular barium (Yamada *et al.*, 1999) reigniting the possibility of potassium mediated presynaptic inhibition. The calcium channel hypothesis has also been favoured by recent studies. Takahashi *et al* have recently shown $GABA_B$ mediated effects on presynaptic calcium influx and transmitter release (Takahashi *et al.*, 1998). The mechanism of presynaptic $GABA_B$ inhibition thus remains controversial.

$GABA_B$ receptors are now known to be metabotropic GABA receptors formed as a dimer of two subunits - $GABA_BR1$ (which occurs in two splice variants: $GABA_BR1a$ and $GABA_BR1b$) and $GABA_BR2$ (Jones *et al.*, 1998; Kaupmann *et al.*, 1998; Kuner *et al.*, 1999; White *et al.*, 1998). They are found both pre- and the post-synaptically (Bowery, 1993) and both synaptically and extrasynaptically (Pham *et al.*, 1998). They are found in both peripheral and nervous tissue, and in the CNS they appear in varied density in most regions of the brain.

4.2.3. $GABA_C$ receptors

A class of GABA receptors which was neither blocked by bicuculline nor activated by baclofen was first described by Drew *et al* (Drew *et al.*, 1984). Following the definitions for GABA receptor types introduced by Hill and Bowery (Hill & Bowery, 1981), these receptors were not $GABA_A$ or $GABA_B$ receptors, and they subsequently have come to be called $GABA_C$ receptors. $GABA_C$ receptor mediated currents were first studied in isolation when bovine retinal cDNA was expressed in xenopus oocytes (Polenzani *et al.*, 1991). Subsequently, they been shown in a variety of mammalian (Feigenspan *et al.*, 1993) and non-mammalian retinal cell types (Dong *et al.*, 1994; Lukasiewicz *et al.*, 1994; Qian & Dowling, 1993; Qian & Dowling, 1994).

$GABA_C$ receptors are thought to be multimers of the ρ type GABA receptor subunits (table 5.2.). These subunits are expressed highly in retinal cells, and when expressed in xenopus oocytes, form functional homomeric and hetermeric ρ receptor GABA receptors which are insensitive to bicuculline (Shimada *et al.*, 1992). These subunits form receptors with an integral chloride selective ion channel which opens in response to GABA or *cis*-aminocrotonic acid (CACA) application (Johnston, 1996). The distribution of the ρ subunits is not limited to the retina however, as all three ρ subunits have been found to be expressed outside the retina (Enz & Cutting, 1999; Wegelius *et al.*, 1998). The ρ subunits appear to combine only with selected non-ρ $GABA_A$ subunits to form functional receptor complexes (Ekema *et al.*, 1998).

4.3. GABA RECEPTOR PHARMACOLOGY

The ionotropic GABA receptors are the target of a wide range of pharmacological agents. More than one hundred ligands have been found to bind to and activate, inhibit or modulate the $GABA_A$ receptor (Johnston, 1996). These agents can be divided into three classes- inhibitors (including competitive and non-competitive

types), agonists, and allosteric modulators. The allosteric modulators exert their effects on the GABA receptor through alteration of the channel opening frequency (Orser *et al.*, 1994; Rogers *et al.*, 1994), or open time (Study & Barker, 1981; Twyman & Macdonald, 1992). These agents are also divided into broad categories, based on the chemical structure and binding site of the agent. Some of these include the benzodiazepines, barbiturates, volatile anesthetics and neuroactive steroids.

4.3.1. $GABA_A$ receptor antagonists

The alkaloid bicuculline was the first GABA receptor antagonist described (Curtis *et al.*, 1970) and antagonism by bicuculline became one of the pharmacological criteria defining the $GABA_A$ class of receptor in 1981 (Hill & Bowery, 1981). Though it is now accepted that bicuculline acts by competing with agonist for the GABA binding site, the actual site of bicuculline interaction has not been resolved (Johnston, 1996). Other competitive antagonists including (in order of potency), RU5135, pitrazepin, SR95531 (gabazine), (+)-hydrastine, securinine, (+)-tubocurarine, and benzylpenicillin are thought to share some structural similarities and thus act at similar if not the same site as bicuculline (Rognan *et al.*, 1992).

The non-competitve antagonists are thought to act at sites close to or within the chloride ion channel of the $GABA_A$ complex. Picrotoxin (an equimolar combination of picrotin and picrotoxinin) is the most widely used of these agents. Radiolabelled analogues of picrotoxinin indicate a binding site close to the chloride channel. Furthermore, binding of a number of other allosteric modulators and $GABA_A$ receptor agonists reduce the binding affinity of radiolabelled picrotoxin analogues (Johnston, 1996) and picrotoxinin can inhibit the $GABA_A$ receptor when administered intracellularly (Akaike *et al.*, 1985). Other non-competitive antagonists include *t*-butylbicyclophosphorothionate (TBPS), *m*-benzenesulfonium diazonium chloride, δ-guanidinovaleric acid, cunaniol, sulfated dopamine metabolites, and furosemide.

4.3.2. $GABA_B$ receptor antagonists

The search for specific potent inhibitors of $GABA_B$ receptors was hindered by the fact that no naturally occurring antagonists had been identified, and was thus directed towards synthetic analogues of GABA and the other known agonist, baclofen. The first recognised selective $GABA_B$ antagonist to emerge from this process was a phosphonic derivative of baclofen, 3-amino-2-(4-chlorophenyl)-propylphosphonic acid (phaclofen) (Kerr *et al.*, 1987). Though specific for $GABA_B$ receptors, phaclofen is only effective at high concentrations (~1 mM (Mott & Lewis, 1994)), though it does show slightly higher potency at post-synaptic $GABA_B$ sites over pre-synaptic (Dutar & Nicoll, 1988). More potent antagonists were found among the sulfonic analogues of baclofen, including saclofen and 2-hydroxy saclofen (Curtis *et al.*, 1988; Kerr *et al.*, 1988). These compounds were an order of magnitude more potent than phaclofen but high concentrations of the antagonists were still required to block agonist binding, and non-specific effects have been reported at these concentrations (al-Dahan *et al.*, 1990).

The most potent antagonists reported to date have been phosphinic analogues of GABA. The first of these, CGP35348, was as potent as 2-hydroxysaclofen, but able to cross the blood-brain barrier (Kerr & Ong, 1995). This compound, like the other phosphinic acid derivatives subsequently reported has no reported non-specific affects at $GABA_A$, glutamate, or a range of other receptors. The most potent of these derivatives, CGP 54626 and CGP 55845 are 5000 times more potent than CGP35348 and 15000 times more potent than phaclofen acting at nanomolar concentrations (Mott & Lewis, 1994).

4.3.3. $GABA_C$ receptor antagonists

Despite being insensitive to the classical $GABA_A$ antagonist bicuculline, $GABA_C$ receptors have been shown to be antagonised by other $GABA_A$ antagonists (SR95531) and agonists (3-aminopropanesulfonic acid (3-APS) and z-3-(amidinothio)propenonic acid (ZAPA)). Similarly, the classical $GABA_B$ agonist baclofen, and antagonists saclofen and phaclofen have no $GABA_C$ activity, whereas

other $GABA_B$ active compounds are quite potent antagonists (3-aminobutylphosphinic acic (3-APPA) and 3-aminopropyl(methyl)phosphinic acid (3-APMPA) (Woodward *et al.*, 1993). The $GABA_B$ agonists 3-APPA and 3-APMPA in particular provided the lead for developing the first specific antagonist of $GABA_C$ receptors – TPMPA (Ragozzino *et al.*, 1996). This compound is a potent inhibitor of retinal $GABA_C$ receptor responses (K_b = 2 μM), and at least 100 times less active at GABA receptors and 500 times less active as an agonist of $GABA_B$ receptors (Murata *et al.*, 1996). Recently however, the sensitivity of $GABA_C$ comprising ρ2 subunits has been shown to be up to 8 times less than that for the homomeric ρ1 $GABA_C$ receptors. The TMPMA sensitivity of $GABA_C$ receptors containing ρ3 subunits as homomers or heteromers with other ρ subunits, has not been studied.

4.3.4. Benzodiazepines

Benzodiazepines have been used therapeutically since the early 1960's as anxiolytics, anticonvulsants, sedative-hypnotics and muscle-relaxants (Dunn *et al.*, 1994). However, it wasn't until the mid to late 1970's that the action of these drugs was shown to be exerted through the potentiation of the inhibitory action of GABA (Costa *et al.*, 1975; Haefely *et al.*, 1975). Subsequent studies indicated this action was the result of these agents binding directly to a benzodiazepine binding site (BZ) integral to the GABA receptor complex (Olsen, 1981). The GABA/BZ receptor, as it then became known, was then classified according to the actions of an expanding range of benzodiazepine site ligands into three types. The first type, BZ_1, demonstrate high affinity modulation by zolpidem, the triazolopyridazine CL218872, and some β-carbolines. BZ_2 receptors show low affinities for these agents, but high affinity for flunitrazepam. Finally, BZ_3 type receptors are insensitive to the classical 1,4-benzodiazepine diazepam but sensitive to the negative allosteric modulator Ro15-4513.

With the cloning of the GABA receptor subunit family (Schofield *et al.*, 1987), a molecular basis for the observed benzodiazepine sensitivities and efficacies emerged (Pritchett *et al.*, 1989). Photoaffinity labeling (Duncalfe *et al.*, 1996), mutation

studies (Amin *et al.*, 1997; Buhr *et al.*, 1997; Buhr & Sigel, 1997; Wieland *et al.*, 1992) and subunit selective expression studies (Pritchett *et al.*, 1989; Pritchett *et al.*, 1989) have indicated that α and γ subunits form the benzodiazepine binding site. The expression studies also demonstrated that it was the different α and γ subunit combinations that form this site which determined the specificity, potency and in some cases effect, of a particular benzodiazepine at a particular GABA receptor type. Receptors containing an α1 subunit in combination with a γ2 subunit were found to exhibit BZ1 type affinity for CL218,872 (Hadingham *et al.*, 1993; Pritchett *et al.*, 1989) whereas receptors containing α2, α3 and α5 subunits are of the BZ2 type, with lower affinity for CL218,872 (Wafford *et al.*, 1992; Wieland *et al.*, 1992). Receptors containing the α4 or α6 subunit fall into the BZ3 class, being insensitive to Diazepam, having low CL218,872 affinity but binding Ro 15-4513 (Wieland *et al.*, 1992). The γ subunit type present also influences the affinity of various benzodiazepines for the binding site. The γ1 subunit when expressed in combination with α and β subunits generally reduces the affinity of these receptors for most benzodiazepines compared to the same combinations containing a γ2 subunit (Whiting *et al.*, 1995). Furthermore, inverse agonists such as DMCM and Ro15-4513 have been shown to act as agonists at receptors containing the γ1 subunit (Puia *et al.*, 1991). The γ3 subunit also confers some lower sensitivities to some benzodiazepines (Herb *et al.*, 1992) but the efficacy of these agents are similar for γ2 and γ3 containing receptors (Whiting *et al.*, 1995). In the light of the fact that the recombinant receptor studies have revealed such an array of benzodiazepine affinities and efficacies, and that no clear typical BZ2 response or molecular combination has been found, the previous BZ 1-3 classification system and the terms benzodiazepine receptor and GABA/Benzodiazepine receptor are now considered outmoded (Barnard *et al.*, 1998). Rather, it is now more pertinent to classify the benzodiazepine based on its affinity and effect at GABA receptors of particular subunit combinations.

Drugs acting at the benzodiazepine site are classified into three types; benzodiazepine agonists, benzodiazepine inverse agonists and benzodiazepine site antagonists. Benzodiazepine agonists are positive allosteric modulators, which include the classical benzodiazepines such as diazepam and flunitrazepam, and those

agents which enhance GABA receptor responses. Recent studies have shown that binding of these agents results in an increase in the frequency of channel opening (Rogers *et al.*, 1994) by increasing the affinity of the receptor for the agonist GABA (Lavoie & Twyman, 1996). Negative allosteric modulators have the opposite effect in reducing GABA receptor responses, and neutralising allosteric modulators block the action of the agonists and inverse agonists by competitively binding to the benzodiazepine site but have no effect on the chloride flux through the GABA receptor (Johnston, 1996; Sieghart, 1995). Also a range of agents which exhibit only intermediate effects compared to the agonist and inverse agonists are collectively called partial agonists and partial inverse agonists. The classification of a benzodiazepine site specific drug into one of these classes is then determined for GABA receptors of a particular subunit combination. The imidazopyridine zolpidem for example, acts as a full agonist at $\alpha_2\beta_3\gamma_2$ and $\alpha_3\beta_3\gamma_2$ but is a partial agonist at $\alpha_5\beta_3\gamma_2$ receptors. Ro15-4513 is a full inverse agonist on $\alpha_5\beta_3\gamma_2$ receptors, a partial inverse agonist on $\alpha_1\beta_2\gamma_2$ receptors and a partial agonist on $\alpha_6\beta_2\gamma_2$ receptors (Whiting *et al.*, 1995).

4.3.5. General anaesthetics

A wide range of structurally unrelated anaesthetic agents have been demonstrated to exert their effects at least partially through the potentiation of GABA receptor responses (Pistis *et al.*, 1997). These anaesthetics include the common inhalational anaesthetics halothane, enflurane, isoflurane, and diethyl ether, and the injectable anaesthetics ketamine, propofol, and alphaxolone. At low concentrations (similar to theraputic doses) these agents have been found to mostly potentiate GABA receptor mediated currents, whereas at higher concentrations these agents activate the chloride current directly (Sieghart, 1995). The effects of these agents does however show variance with different receptor subunit compositions (Lin *et al.*, 1993; Sanna *et al.*, 1995). GABA receptors comprising α1, α2 and α5, β1 and β2 and γ2 (S and L) subunits show positive modulation (Sanna *et al.*, 1995), whereas receptors comprising α6 subunits are less sensitive (Krasowski *et al.*, 1997), and those containing the ε subunit are insensitive (Davies *et al.*, 1997). The direct activation of

the GABA receptor by higher concentrations of anaesthetic appears to require a β subunit indicating that this action may occur at an alternate binding site (Belelli *et al.*, 1996; Sanna *et al.*, 1995). $GABA_C$ receptors which are combinations of the ρ1 subunits, are inhibited by the inhalational anaesthetics enflurane, halothane and isoflurane but unaffected by other anaesthetic agents (alphaxolone, propofol) (Mihic & Harris, 1996) and glycine receptors have also been shown to be positively modulated by anaesthetics (Pistis *et al.*, 1997). These results may indicate binding domains for these agents in conserved transmembrane regions of $GABA_A$, $GABA_C$ and glycine receptors (Mihic *et al.*, 1997) though the site for each agent may not be the same (Krasowski *et al.*, 1998).

Propofol has been demonstrated to positively modulate recombinent GABA receptors containing combinations of α,β and γ subunits by varying degrees depending on the combination (Sanna *et al.*, 1995). However, inclusion of the recently cloned ε subunit into an α/β combination confers propofol insensitivity to these receptors. Propofol has also been reported to be ineffective at homomeric ρ1 $GABA_C$ receptors (Mihic & Harris, 1996), though its effect on other forms of $GABA_C$ receptors has not been investigated.

Used at concentrations close to is therapeutic dose (9.2 - 56 μM in an anaesthetised patient's serum), propofol has been demonstrated to enhance $GABA_A$ mediated inhibition in pyramidal hippocampal neurones from region CA1 (Albertson *et al.*, 1996). Furthermore, the effect of propofol on miniature spontaneous IPSC recorded from cultured hippocampal neurones was to elongate the decay of these events while not affecting peak amplitude (Orser *et al.*, 1994). In the same study, single channel recordings made from outside-out patches from the same cultured neurones indicate that at low concentration, propofol increases the frequency of GABA receptor channel opening in the presence of agonist.

4.3.6. Barbiturates

Like the general anaesthetics described above the barbiturates (pentobarbital, pentobarbitone, phenobarbital and secobarbital), have also been demonstrated to

have both allosteric modulatory effects and direct activation effects (Sieghart, 1995). These effects are also somewhat subunit dependant with the α subunit important for efficacy of the barbiturate induced potentiation and the direct activation at higher concentrations (Thompson *et al.*, 1995). In contrast, homomeric ρ1 $GABA_C$ receptors have been shown to be unaffected by pentobarbital (Mihic & Harris, 1996; Shimada *et al.*, 1992), however the sensitivity to and effect of barbiturate on other $GABA_C$ receptor subunit compositions has not been reported.

4.4. GLYCINE

The amino acid glycine was also shown to exhibit inhibitory actions on spinal neurones in the early 1960s (Curtis *et al.*, 1961; Curtis *et al.*, 1959). Subsequently, glycine has also been shown to meet all of the criteria of chemical transmitters and to act as an inhibitory transmitter (Van den Pol & Gorcs, 1988). Fast ionotropic glycinergic transmission has been demonstrated in the caudal regions of the CNS – the brainstem and spinal cord (Kotak *et al.*, 1998; Lewis & Faber, 1996; Schneider & Fyffe, 1992), though the mRNA for the glycine receptor subunits (α1, α2, and β) have been shown to be expressed throughout the brains (Sato *et al.*, 1992). In the brainstem and spinal cord, the activation of glycine receptors was shown to activate a chloride conductance (Curtis *et al.*, 1968) – by opening an ion channel which is formed through the receptor complex (Betz, 1992). The responses of the neurones is an acute hyperpolarisation similar to that recorded in response to the activation of the ionotrophic GABA receptors and the mechanism of glycinergic inhibition is thus assumed to be the same as that of fast ionotropic GABAergic inhibition (Curtis *et al.*, 1968).

4.5. GLYCINE RECEPTORS

Belonging to the same superfamily of ligand gated ion channels as acetylcholine and GABA receptors, the glycine receptor is also thought to consist of a pentameric assembley of glycine receptor subunits (Betz, 1992; Langosch *et al.*, 1990). To date, three α subunits (α1,α2, and α3) and 1β subunit have been cloned in rat, with the α2 subunit also having three splice variants (α2A,α2B, and α2*) and the α1 having a variant with an eight amino acid extension at its C-terminus ($\alpha1^{ins}$) (Rajendra *et al.*, 1997). Functional glycine receptors are composed of a combination of either α subunits alone, or in combination with β subunits. The subunit combination which forms the expressed glycine receptor is developmentally regulated, such that the fetal glycine receptor is a pentomer of α2 subunits and the adult form is a combination of the α1 and β subunits (Rajendra *et al.*, 1997). Like the GABA receptor, the glycine receptor complex has an integral ion channel through the complex structure which is permeable to chloride ions and other monovalent anions (Rajendra *et al.*, 1997).

Glycine receptors and mRNA for the subunits, have been localised throughout the CNS in rats (Betz, 1991; Sato *et al.*, 1992; Van den Pol & Gorcs, 1988), however immunoreactivity to both the receptor and to glycine itself is diffuse outside the spinal cord and brainstem. Furthermore, synaptic activation of these receptors appears to be limited to synapses in brainstem and spinal cord, as GABA is thought to solely mediate fast inhibitory transmission in the rostral regions of the CNS (Flint *et al.*, 1998). The role of the glycine receptors in the brain has not been established. Reports of non-synaptic activation of neocortical glycine receptors by the amino acid taurine during development (Flint *et al.*, 1998) and effects of focally applied glycine on synaptic transmission in the cortex and hippocampus (Bernardi *et al.*, 1979; Ito & Cherubini, 1991), may suggest non-synaptic inhibitory or developmental functions.

4.5.1. Glycine receptor antagonists

The convulsant alkaloid strychnine is a potent competitive inhibitor of the glycine receptor responses (Curtis *et al.*, 1968) binding to a site on the α subunits (Betz, 1992). The IC_{50} for the strychnine block of the glycine receptor is 28 – 49 nM (Jonas *et al.*, 1998; Shirasaki *et al.*, 1991). Picrotoxin also inhibits the glycine receptor. Unlike the noncompetive block of the $GABA_A$ receptor, picrotoxin is a competitive antagonist at glycine receptors, with slightly higher affinity for α1 homomeric receptors over α1β heteromers (Rajendra *et al.*, 1997).

4.6. RESULTS

4.6.1. Ce neurones express both GABA and glycine receptors

Individual Ce neurones responded to applications of both GABA and glycine (n = 7/7) when applied separately by bath application, indicating expression of both Glycine and GABA receptors on these neurones. However, as a result of the relatively slow application method, these responses were quite variable from one application to the next. Respones to GABA and glycine were subsequently studied separately using iontophoretic application of the transmitter. This method enabled repeated application of transmitter at a constant concentration, and hence uniform responses could be evoked. Caesium based internal solutions were used to block potassium channels to isolate $GABA_A$ responses in the presence of putative $GABA_B$ receptors on these cells.

Glycine iontohporesis

Glycine was applied iontophoretically to eleven Ce neurones and responses recorded in increasing concentrations of strychnine. In control recordings (no strychnine) using high chloride internal solution, these responses reversed at 0 mV (Figure 4.1.A,B), and appeared as inward current deflections at membrane potential of –60

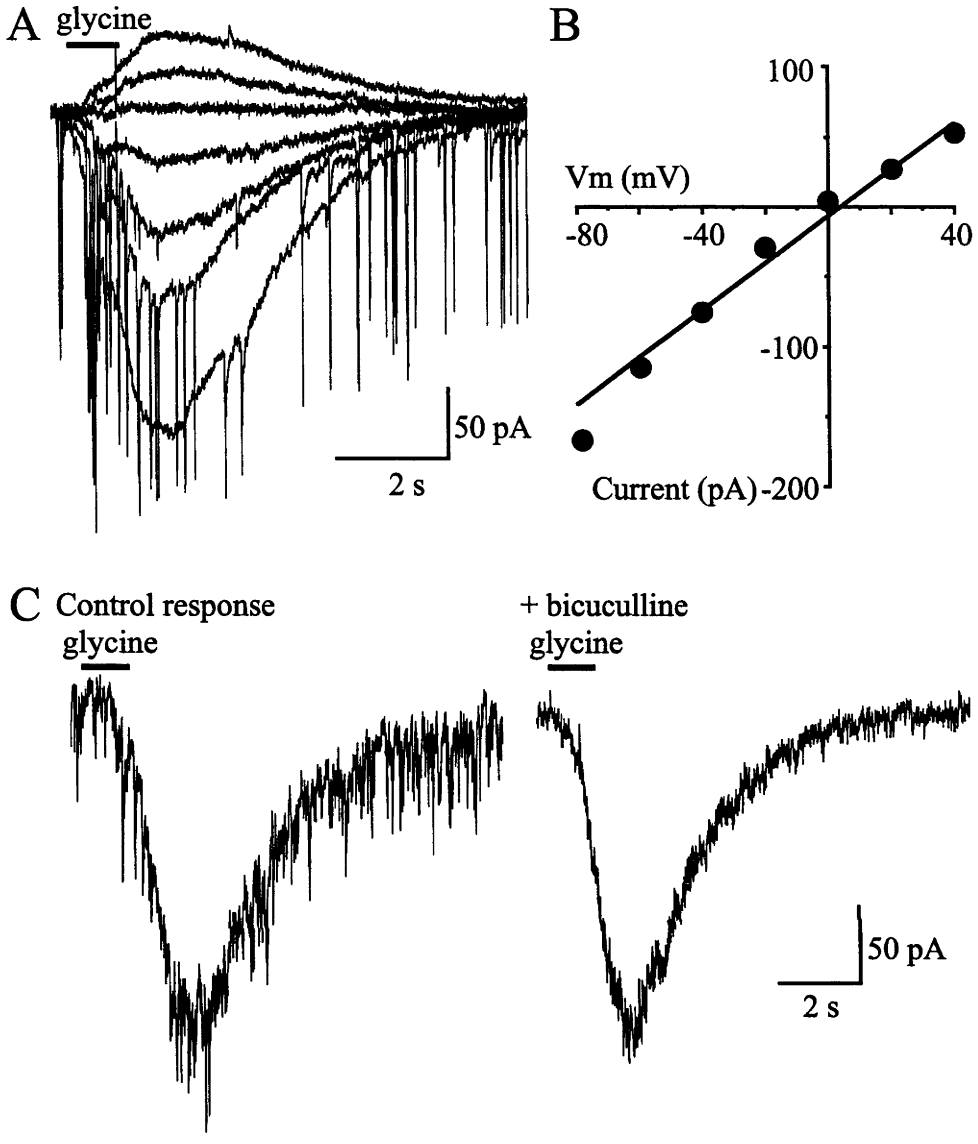

Figure 4.1. Application of glycine to Ce neurones by iontophoresis
A. Responses to iontophoretically applied glycine (duration indicated by bar) at membrane potentials -80, -60, -40, -20, 0, +20 and +40 mV, recorded using cesium chloride internal solution. B. Current voltage relationship for iontophoretically induced glycine current for neurone shown in A. C. Glycine application at Vm -60 in control and in 10 μm bicuculline where spontaneous activity associated with the control response has been abolished.

mV. Notably, these responses to applied glycine were often accompanied with an increase in the frequency of spontaneous synaptic activity (Figure 4.1.C). This activity was sensitive to the $GABA_A$ antagonist bicuculline (Figure 4.1.C), indicating that the increase in release frequency occurred at GABAergic terminals. The response current to iontophoretic glycine was not blocked by 10 μM bicuculline (Figure 4.1.C), but it as blocked in a dose dependent manner by strychnine (Figure 4.2.A). Combining results from six neurones, a dose response curve for the strychnine block of the glycine current (Figure 4.2.B), revealed that the IC_{50} for this block was 0.079 μM, which is close to previously reported IC_{50} for the action of strychnine on glycine receptors on isolated cells and membrane patches (Jonas *et al.*, 1998; Shirasaki *et al.*, 1991).

GABA iontophoresis

Iontophoresis of GABA also produced response currents which reversed at the chloride equilibrium potential (Figure 4.3.A,B). These currents were insensitive to the glycine receptor antagonist strychnine at 1 μM (1.44 ± 7.2% inhibition, n=3) (Figure 4.3.C). The GABA responses were sensitive to the $GABA_A$ antagonists bicuculline and SR95531 however. Recording responses in increasing concentrations of bicuculline (Figure 4.4.) revealed a biphasic block of the responses, with an initial effect between concentrations of 0.05 and 1 μM followed by a second rapid phase of inhibition between concentrations of 10 and 100 μM. This biphasic block was apparent on the inhibition curve constructed from data from six neurones, with the data being best fit by the sum of two sigmoid functions using the equation;

$$\%\text{Inhibition} = a/(1+(IC_{50low}/c)^2) + b/(1+(IC_{50high}/c)^2)$$

where (a + b) was constrained to 100. The IC_{50low} and IC_{50high} represent the IC_{50} for a low affinity binding site and high affininty binding site. IC_{50high} of 0.12 μM was close to that reported by Jonas *et al* for bicuculline inhibition of $GABA_A$ receptors on patches pulled from spinal motorneurones (Jonas *et al.*, 1998). The low affinity bicuculline binding site had an IC_{50} of 23.1 μM, which is twenty times higher than reported at spinal cord $GABA_A$ receptors (Jonas *et al.*, 1998; Shirasaki *et al.*, 1991).

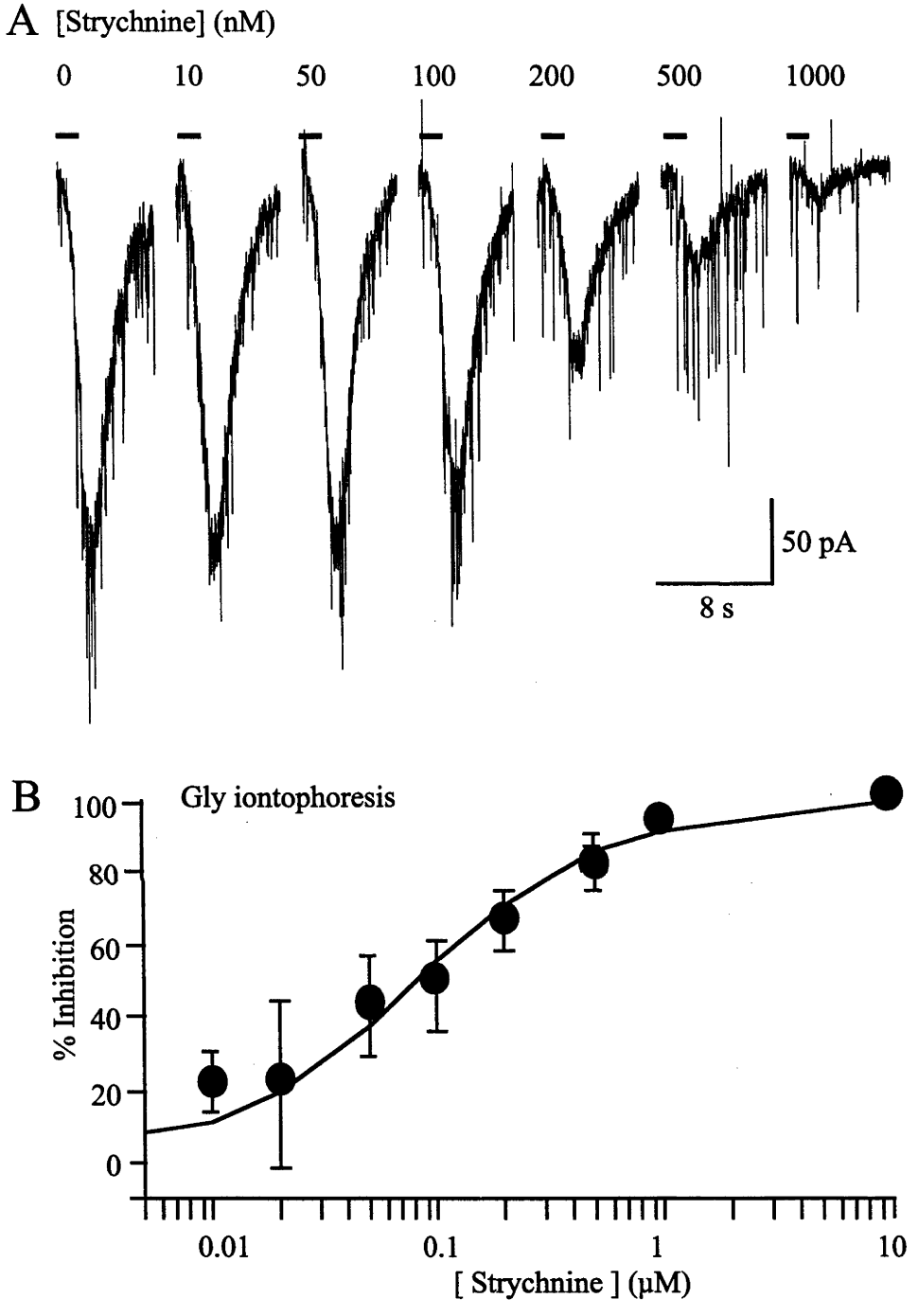

Figure 4.2. Inhibition of glycine currents by strychnine
A. Reponses to iontophoretically applied glycine (duration indicated by bars) in increasing concentration of strychnine (0 - 1 µM). B. Inhibition curve for six neurones showing line of best fit of the function $1/(1+1/(IC_{50}/c))$, where the IC_{50} was 0.079 µM.

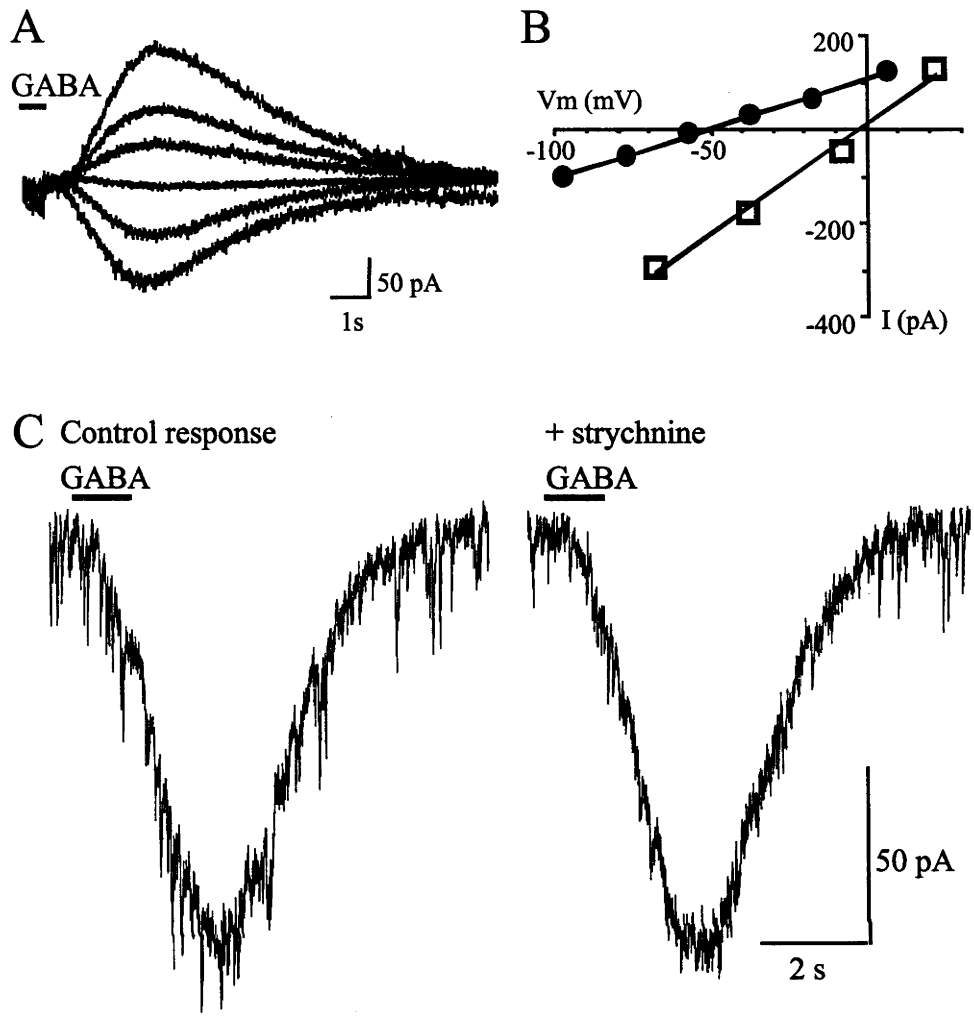

Figure 4.3. Application of GABA to CeL neurones by iontophoresis.
A. Responses to iontophoretically applied GABA (duration indicated by bar) at membrane potentials -100, -80, -60, -40, -20, and +10 mV, using cesium gluconate based internal (E_{Cl} = -50 mV). B. Current-voltage relationship for the recordings shown in A. (filled circles) and for recordings from another neurone using cesium chloride based internal solution (open squares). Both relationships were fit with a linear function passing through the reversal potential for chloride for the internal used (E_{Cl} = -50 mV for cesium gluconate based internal and 0 mV for cesium chloride based internal).
C. Response to iontophoretically applied GABA, recorded in control at Vm -60 and with 1 μM strychnine in the extracellular solution.

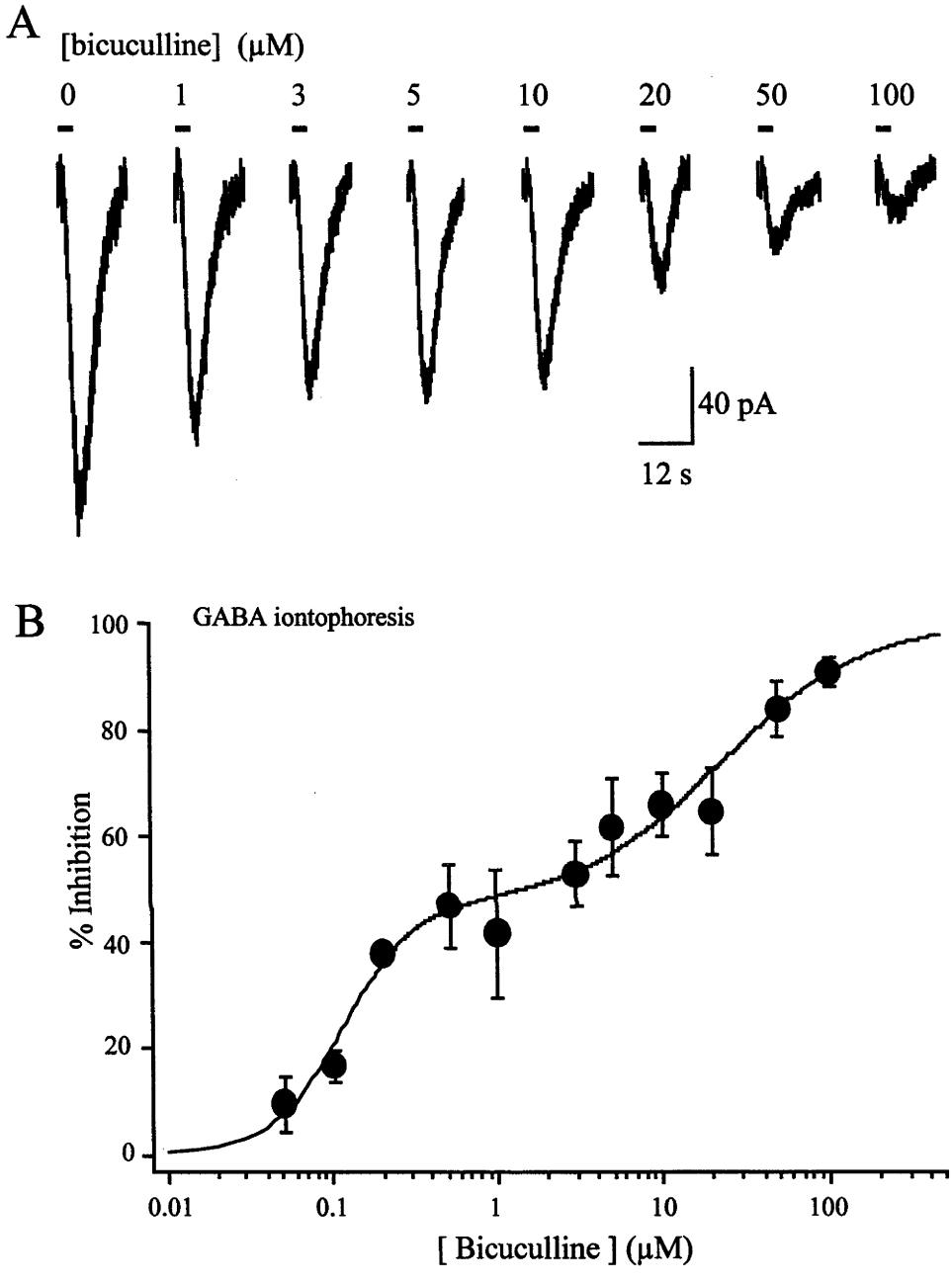

Figure 4.4. Inhibition of GABA currents by bicuculline.
A. Average responses to iontophoretically applied GABA (duration indicated by bars, average of six applications) in increasing concentrations of bicuculline (0 - 100 μM). B. Inhibition curve for responses from six neurones (n = 3 - 6 for each point except 0.2 μM, where n = 2) showing biphasic fit (equation $a/(1/(1+(IC_{50}/c)^2)+b/(1+(IC_{50}/c))$, where a+b was constrained to equal 1) and IC_{50} values of 0.12 μM and 23.1 μM.

As the action of bicuculline at the $GABA_A$ receptor is that of a competitive antagonist, it is possible that in the presence of higher concentrations of the agonist, the action of the antagonist may be affected by increased competition for the binding site. To address the possibility that the second phase of bicuculline inhibition was a secondary block at sites where the bicuculline had been 'competed off' by GABA application, we repeated the experiment using the higher affinity competitive antagonist SR95531. The iontophoretic responses recorded in increasing concentrations of SR95531 (Figure 4.5.A) showed a similar biphasic course of inhibition as the block by bicuculline. The combined results from four neurones (Figure 4.5.B) also provided an inhibition curve that was best fit using the sum of two sigmoid functions (see above). This fit again provided high affinity and low affinity IC_{50} of 0.01 and 0.62 μM respectively. The IC_{50} for SR95531 block of a GABA mediated IPSC has not been reported, however displacement studies using various GABA receptor ligands (muscimol, [^{3}H]GABA) have reported the K_b for SR95531 as between 6.6 nM and 140 nM (Hamann *et al.*, 1988; Ito *et al.*, 1992; McCabe *et al.*, 1988). Though the actual affinity of SR95531 for the GABA receptor is unclear, it has consistently been found to be 14 - 20 times more potent than bicuculline in these studies (Dunn *et al.*, 1994; Ito *et al.*, 1992; Yu & Ho, 1990). This difference in potency is also observed for the high affinity block of the iontophoretic responses by bicuculline and SR95531 in this study. This may indicate that the high affinity component of the bicuculline and SR95531 block seen here results from the binding of these antagonists to the same GABA receptor binding sites.

The presence of a low affinity antagonist site indicated the presence of a second GABA receptor type which was less sensitive to bicuculline and SR95531 block. The $GABA_C$ class of receptor have been shown to be at most only weakly inhibited by high doses of bicuculline (>100 μM), but partially sensitive to SR95531 (Feigenspan & Bormann, 1994; Woodward *et al.*, 1993) albeit at 20 times the dose required for $GABA_A$ inhibition ((Woodward *et al.*, 1993). $GABA_C$ receptors are blocked by a selective competitive antagonist (1,2,5,6-tetrahydorpyridine-4-yl)methylphosphinic acid (TPMPA) (Chebib *et al.*, 1998; Ragozzino *et al.*, 1996) with an IC_{50} of approximately 0.7 μM (K_b = 2μM) for homomeric ρ1 $GABA_C$

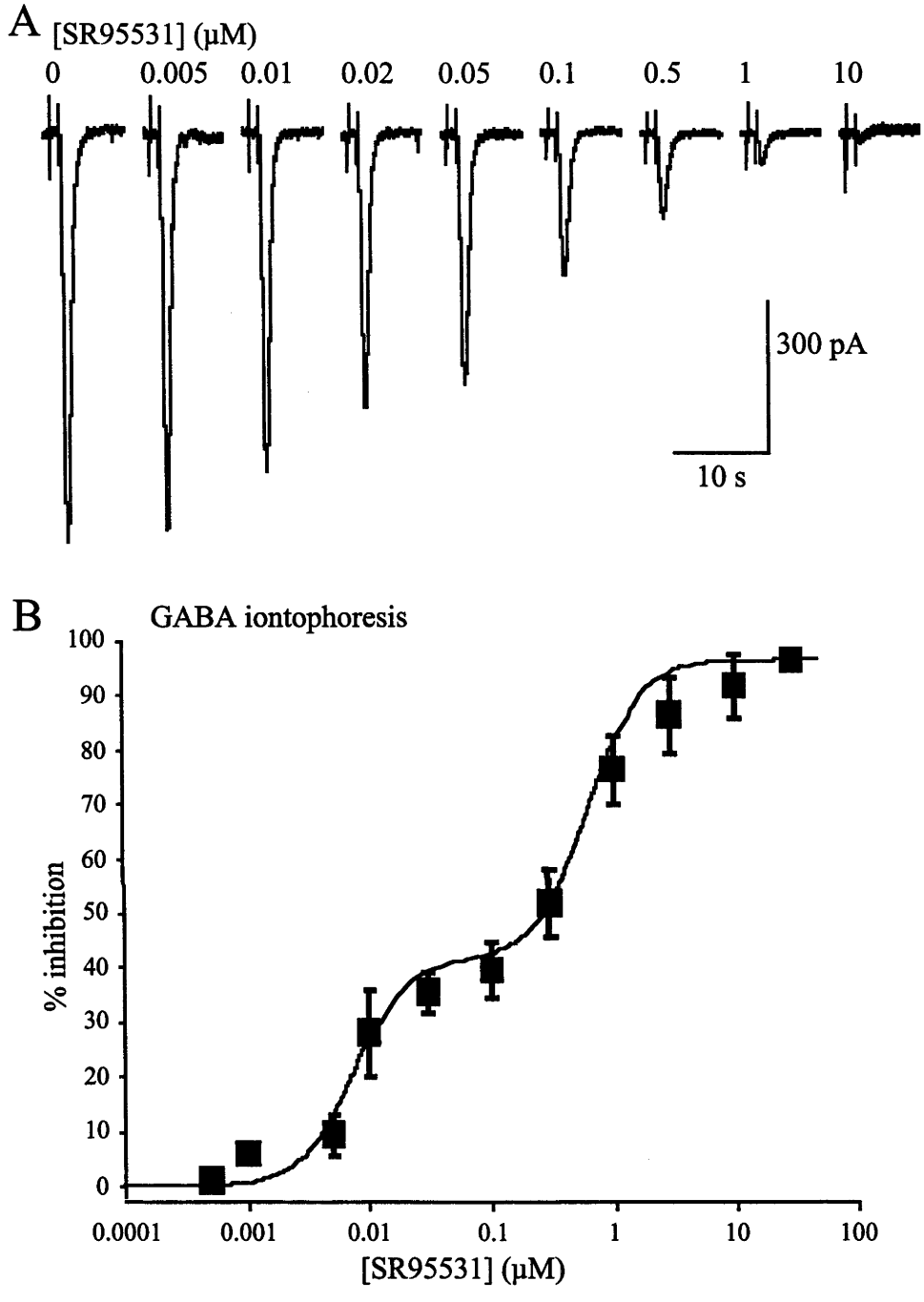

Figure 4.5. Inhibition of GABA currents by SR95531.
A. Average responses to iontophoretically applied GABA (six 100 ms applications) in increasing concentration of SR95531 (0 - 10 μM).
B. Inhibition curve for data from six neurones (n = 3 - 4 for each point except 0.0005, 0.001,and 30 μM where n = 1, 2 and 2 respectively) showing biphasic fit (equation $a/(1/(1+(IC_{50}/c)^2)+b/(1+(IC_{50}/c))$, where a+b was constrained to equal 1) and IC_{50} values of 0.008 μM and 0.622 μM.

receptors (Ragozzino *et al.*, 1996) but eight times less potent at homomeric ρ2 $GABA_C$ receptors (K_b = 16 μM) (Chebib *et al.*, 1998). To test the TPMPA sensitivity of the component of the GABA response blocked by the low affinity binding of bicuculline and SR95531, TPMPA was applied at 60 μM to iontophoretic responses in the presence of 10 μM bicuculline (Figure 4.6.). This dose of bicuculline has been used routinely to block $GABA_A$ mediated inhibition in previous studies (Collingridge *et al.*, 1984; Singer & Berger, 1999; Williams & Johnston, 1991) and would block the contribution of the high bicuculline affinity receptors by more than 99% (assuming an IC_{50} of 0.12 μM as described above). The iontophoretic currents in the presence of bicuculline were found to be reversibly blocked by 73% ± 1% by 60 μM TPMPA. These results indicate that the low bicuculline affinity site is also a binding site for the selective $GABA_C$ antagonist TPMPA and indicates the presence of a second GABA receptor with $GABA_C$-like antagonist sensitivity on Ce neurones.

4.6.2. GABA receptors mediate the fast IPSC in Ce neurones

The presence of all three known chloride channel ionotropic receptors ($GABA_A$, glycine and a putative $GABA_C$ receptor) on neurones of the Ce provided a number of possibilities for the composition of the synaptic inhibitory currents recorded in these neurones. Co-release of glycine and GABA has been demonstrated at synapses containing both glycine and $GABA_A$ receptors (Jonas *et al.*, 1998,O'Brien, 1999 #2127) which results in composite IPSC. In contrast, hippocampal pyramidal neurones which have been shown to express both glycine receptors (Sato *et al.*, 1992) and $GABA_A$ receptors, have IPSC which are mediated by GABA receptors only (Segal & Barker, 1984). Though the expression of $GABA_A$ and $GABA_C$ receptors on retinal neurones has been demonstrated both histologically (Enz *et al.*, 1996; Fletcher *et al.*, 1998) and pharmacologically (Feigenspan *et al.*, 1993; Qian & Dowling, 1993), only a few papers have demonstrated synaptic currents mediated by the $GABA_C$ receptors (Hartveit, 1999; Lukasiewicz & Shields, 1998). From these, no clear picture has emerged of the contribution of $GABA_C$ and $GABA_A$ currents to IPSC where both receptors may be activated.

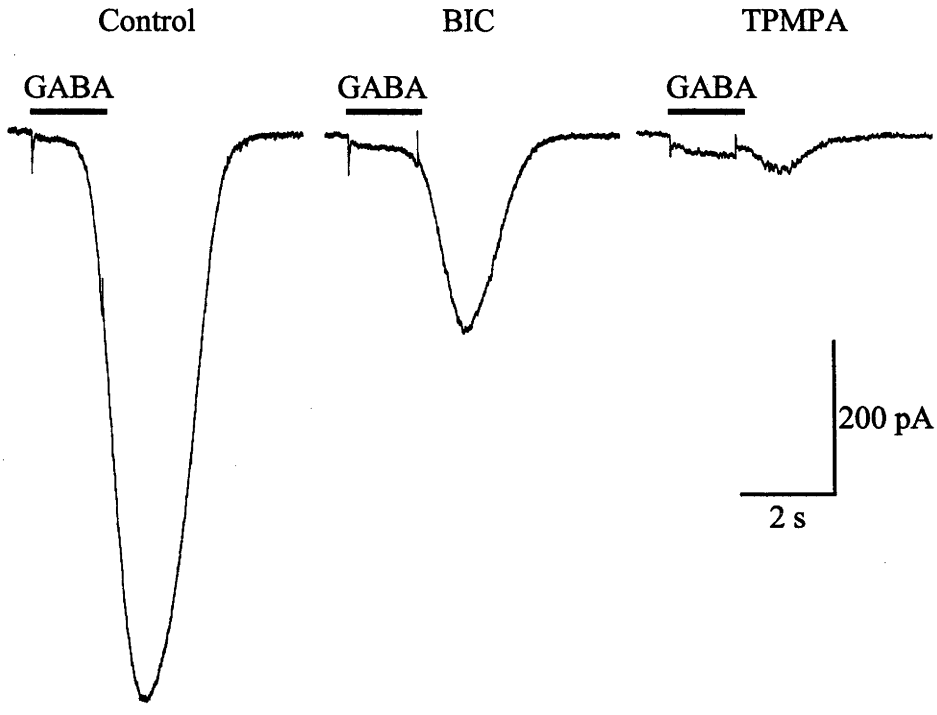

Figure 4.6. TPMPA blocks the iontophoretic GABA response insensitive to 10 µM bicuculline.
Iontophoretic responses to GABA, partially blocked by 10 µM bicuculline (BIC) were further blocked on application of 60 µm TPMPA (TPMPA). Responses shown are averages of six individual iontophoretic applications.

To study the receptor types mediating the IPSC in the Ce we evoked electrically monosynaptic inputs from the ICM region lying laterally to the CeL, and recorded from neurones within the CeL. The IPSC was isolated by blocking the glutamatergic EPSC, using either kynurenic acid (2 mM) or a combination of CNQX (10 μM) and D-APV (30 μM). To determine whether $GABA_A$ receptor mediated current was contributing to the evoked IPSC, we applied bicuculline (10 μM) to the extracellular solution. The control IPSC was blocked by on average 66.9 ± 3.3% (n = 19) by bicuculline at this concentration (Figure 4.7.A,C). Assuming that the synaptic GABA receptors are the same as those activated by the iontophoresis of GABA onto these cells, the effect of 10μM GABA would be to block the high bicuculline affinity $GABA_A$ receptors by more than 98.5%. This was also the case for the $GABA_A$ mediated IPSC recorded in hippocampal pyramidal neurones in region CA1, which were blocked by 98.3 ± 0.6% as a control experiment (Figure 4.7.B,C). Partial block of the IPSC by this concentration of bicuculline thus indicated a second contributing receptor to the IPSC.

Evoked IPSC responses were isolated in either kynurenic acid (2 mM) or CNQX and D-APV (10 and 30 μM respectively), and 10 μM bicuculline, to study the receptor underlying the non-$GABA_A$ component of the IPSC. The resultant IPSC recorded had a reversal potential similar to the control IPSC in both cesium chloride and caesium gluconate internals, at close to the reversal potential for chloride using these internals (0 and –52 mV respectively) (Figure 3.7.A,B). This indicated that the second IPSC component was a chloride current.

The selective glycine antagonist strychnine was applied to test whether the glycine receptors were mediating the second IPSC component. When applying glycine by iontophoresis, 1 μm strychnine had blocked the glycine receptor response by more than 98%. However, this concentration had no significant effect on the IPSC recorded in the presence of 10 μM bicuculline (0.35 ± 2.1%, n = 3) (Figure 4.8.). This indicated that the glycine receptors were not contributing to the IPSC. Furthermore, the addition of the GABA uptake inhibitor NO-711 (1 μM) was able to prolong the decay of a train of IPSCs recorded in the presence of 10 μM bicuculline

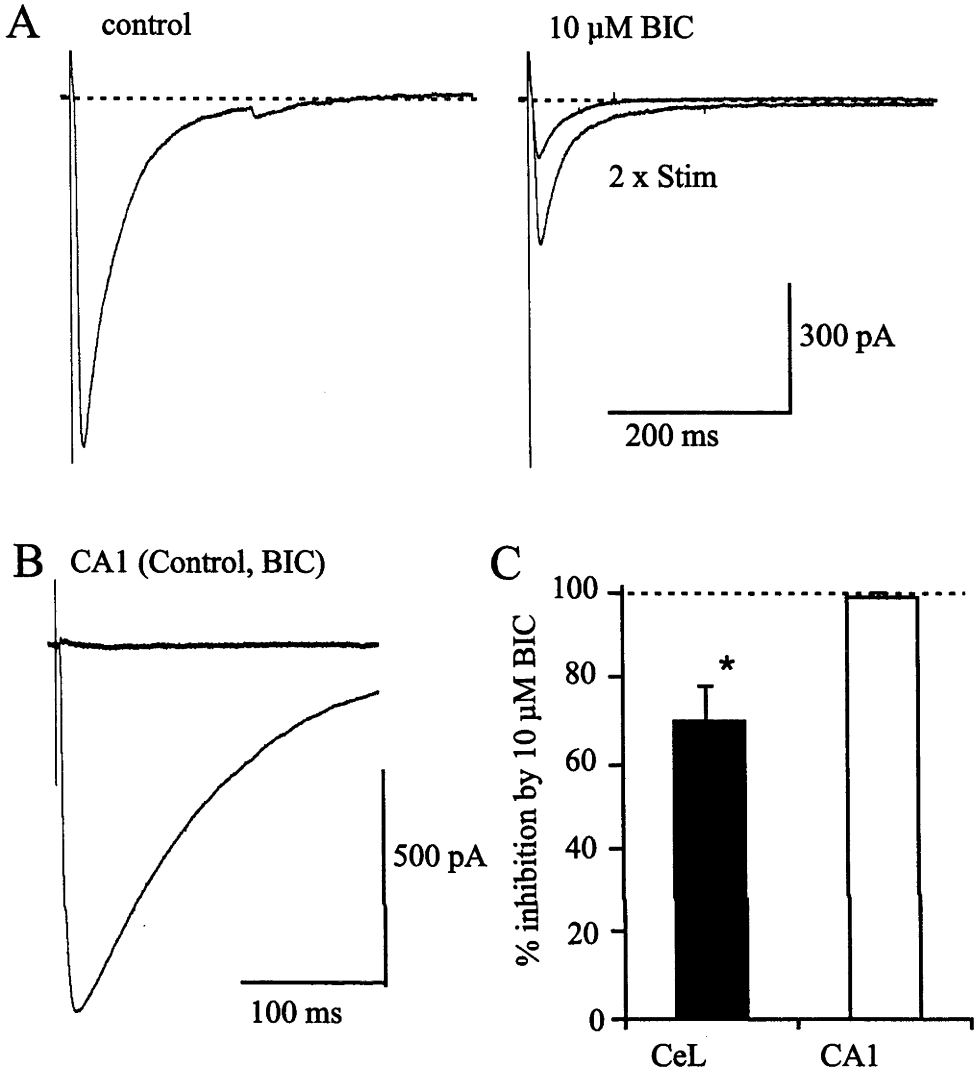

Figure 4.7. The CeL IPSC is partially blocked by 10 µM bicuculline.
A. IPSC were evoked electrically, laterally to CeL in the presence of kynurenic acid (2 mM). Bicuculline (10 µM) applied in the extracellular solution partially blocked the IPSC, leaving a component which increases with increased stimulation strength. B. CA1 IPSC in control (light line) and blocked by 10 µM bicuculline (heavy line). C. The average effect of 10 µM bicuculline on IPSC recorded in CeL compared to IPSC recorded in pyramidal neurones from the hippocampal CA1 region. ($p < 0.05$, $n = 19$).

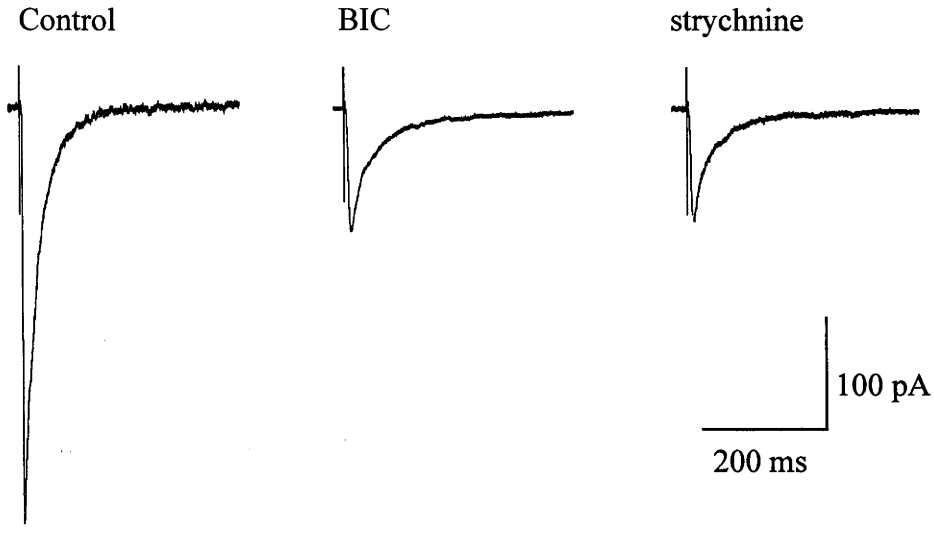

Figure 4.8. Strychnine has no effect on the bicuculline insensitive IPSCs. IPSCs were evoked electrically laterally to CeL (control) and bicuculline (10 μM) added to block the $GABA_A$ component (BIC). The remaining IPSCs were unaffected by strychnine (1 μM). (Average of six individual IPSCs shown).

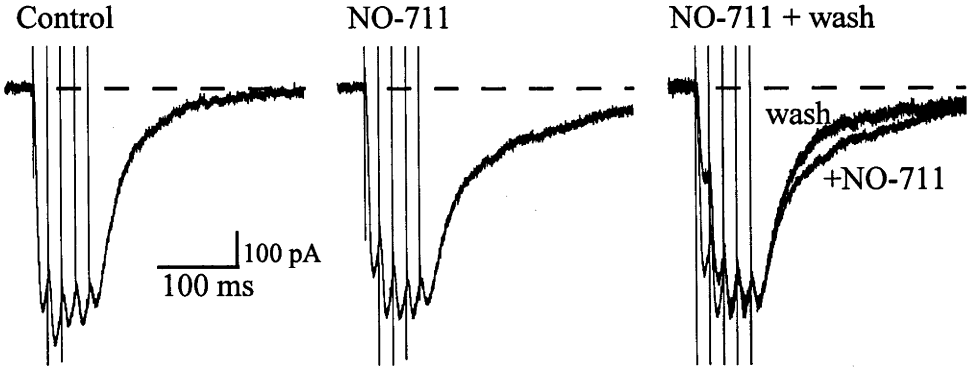

Figure 4.9. The GABA uptake inhibitor NO-711 prolongs the IPSC decay. A IPSCs produced by a train of stimuli (5 stimuli at 50 Hz) in control (2mM kynurenic acid), in the presence of extracellular NO-711 and this response overlaid on IPSCs after washout of NO-711. (Averages of six individual IPSCs in each case.)

(Figure 4.9.), indicating that these responses were activated by synaptic GABA release.

To confirm that the second GABA receptor type identified by the GABA iontophoresis was contributing to the IPSC, the effects of higher concentrations of bicuculline and the $GABA_C$ antagonist TPMPA were examined on the component of the IPSC remaining in 10 μm bicuculline. The component of the IPSC left in 10 μM bicuculline was significantly blocked by 60 μM TPMPA (66.2 ± 5.8%, n = 6, $p < 0.01$) (Figure 4.10.A,D). As a control experiment, the same dose applied to hippocampal pyramidal cell IPSC (mediated by $GABA_A$ receptors only) had no significant effect (3.3 ± 4.3% block, n = 7) (Figure 4.10.C,D). Finally, applying TPMPA to the control CeL IPSC partially blocked the responses (36.8 ± 1.8%, n = 6, $p < 0.05$), leaving a proportion of the IPSC similar to that blocked by bicuculline, which could be subsequently blocked by bicuculline (Figure 4.10.B,D).

The block by bicuculline of the iontophoretic GABA response had indicated two IC_{50} for this antagonist, with the effect of higher concentrations (50 – 100 μM) being close to complete block. As seen for these iontophoretic GABA responses, the effect of a higher concentration of bicuculline on the IPSC was also a greater block (86.7 ± 2.9%, n = 4) (Figure 4.11.), but again not complete.

The component of the IPSC not blocked by 10 μm bicuculline mirrored the pharmacological profile of the low bicuculline affinity receptors identified by iontophoresis. Furthermore, the transmitter producing these bicuculline resistant IPSC was identified as GABA by blocking GABA uptake. These results taken together, indicated that the low bicuculline affinity GABA receptors were also contributing to the evoked IPSC.

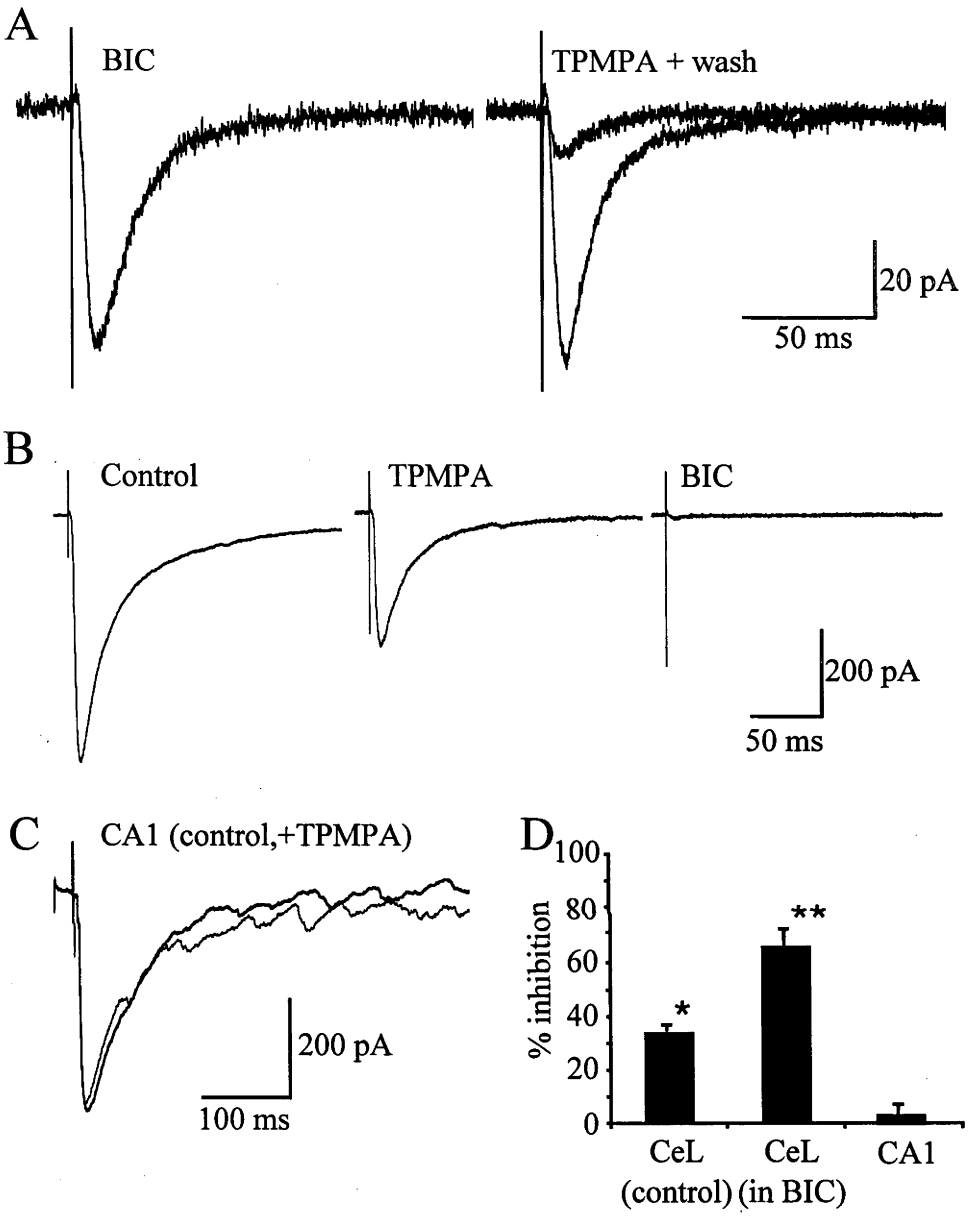

Figure 4.10. TPMPA blocks the IPSCs left in bicuculline.
A. TPMPA (60 μM) was applied to laterally stimulated IPSCs in 10 μM bicuculline (BIC). The bicuculline resistant IPSCs were reversibly blocked (TPMPA + wash). B. A proportion of the control IPSCs similarly stimulated (Control) were also sensitive to TPMPA (60 μM) leaving IPSCs (TPMPA) which were blocked by bicuculline (BIC). C. CA1 IPSCs recorded in control (light line) and TPMPA (heavy line). D. The average effect of TPMPA on control IPSCs, and IPSCs resistant to 10 μM bicuculline in CeL neurones, and the effect of the same dose of TPMPA on IPSCs recorded in hippocampal pyramidal neurones from area CA1.
(* $p < 0.05$, ** $p < 0.01$, $n = 6$ for both experiments). (Average IPSCs shown)

4.6.3. The low bicuculline affinity GABA receptors are less sensitive to picrotoxin than $GABA_A$ receptors

Both $GABA_A$ and $GABA_C$ receptors have been found to be blocked by picrotoxin, however the $GABA_C$ receptors have been shown to be less sensitive (Feigenspan *et al.*, 1993; Qian & Dowling, 1994). Picrotoxin applied at 25 μM was found to completely block the $GABA_A$ mediated IPSC recorded in hippocampal pyramidal neurones in region CA1 (99.1 ± 0.3%, n = 4) (Figure 4.12.A,C). In contrast, the same dose only blocked the control CeL IPSC by 63.6 ± 9.1% (n = 3) (Figure 4.12.B,C), and a much higher concentration (100 μM) was required for near complete block 96.7 ± 0.3 (Figure 4.12.B).

4.6.4. The actions of 1,4-benzodiazepines on the CeL GABA receptors

The homomeric ρ1 $GABA_C$ receptor (and presumably the heteromers containing the ρ2 and ρ3 subunits) are not modulated by benzodiazepines as the binding of these drugs occurs at a site within the $GABA_A$ α and γ subunits. Sensitivity of the bicuculline resistant GABA receptor to the 1,4-benzodiazepine was investigated to establish if these receptors also lacked this site. Applied to the control CeL iontophoretic GABA response (no bicuculline), diazepam produced an insignificant increase in the response amplitude of 21.1 ± 18.8% (n = 5, Figure 4.13.A,D), Uniform control iontophoretic GABA responses were recorded in the presence of bicuculline (10 μM) and Diazepam (10 μM). In contrast to the control CeL responses, responses to applied GABA in the presence of bicuculline were reduced in amplitude by diazepam (Figure 4.13.B) by an average of 27.3 ± 8.1% ($p < 0.05$, n = 5, Figure 4.13.D). A control experiment using the same concentration of diazepam on GABA responses mediated solely by $GABA_A$ receptors on pyramidal hippocampal neurones in region CA1 (Figure 4.13.C) resulted in an increase in the response amplitude by an average of 51.7 ± 19.1% ($p < 0.05$, n = 5, Figure 4.13.D). The small effect of diazepam on the control CeL GABA response represents the net effect of diazepam modulation of both GABA receptor types, ie. the sum of the negative modulation of the bicuculline resistant type and the positive modulation of

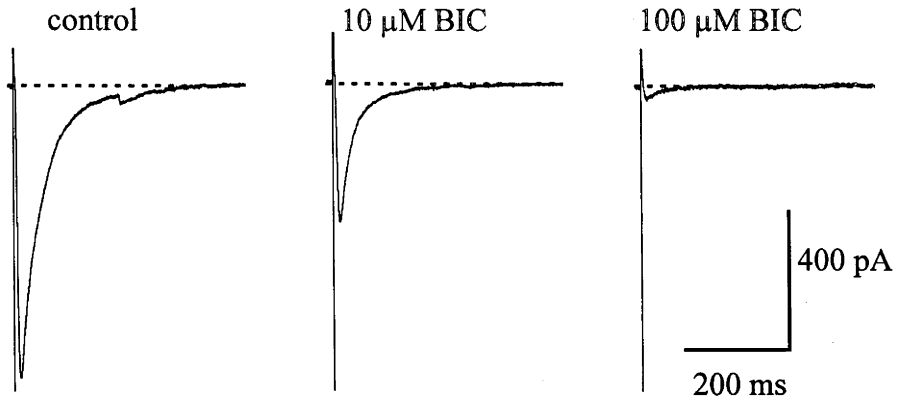

Figure 4.11. Higher Doses of bicuculline block the CeL IPSCs.
IPSCs were evoked electrically laterally to CeL (Control*) and partially blocked by 10 μM bicuculline (10μM BIC*). The remaining IPSCs were further blocked by a higher concentration of bicuculline (100 μM BIC*). (*Averages of six individual IPSCs.)

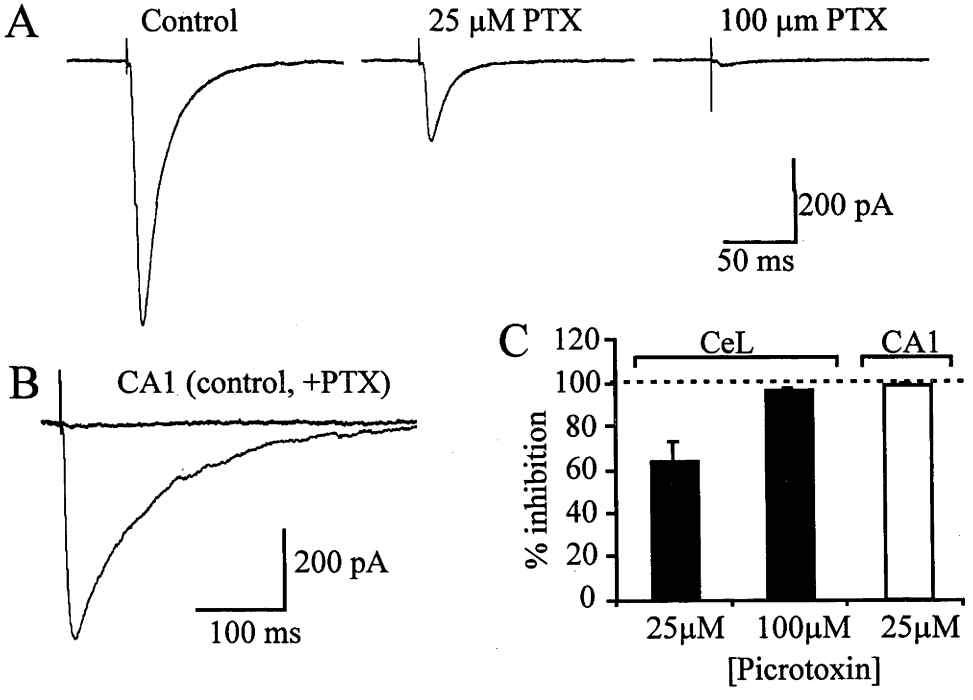

Figure 4.12. CeL IPSCs are less sensitive to picrotoxin than CA1 IPSCs.
A. CeL IPSCs evoked by lateral stimulation (Control*) are partially blocked by 25 μM picrotoxin (25 μM PTX*), but further reduced by higher concentrations (100 μM PTX*). B. CA1 IPSC recorded in control (light line*) and blocked by 25 μM PTX (heavy line*). C. Average block by 25 and 100 μM picrotoxin of laterally stimulated CeL IPSC, compared with complete block of IPSC recorded in CA1. (*Averages of six or more individual IPSCs)

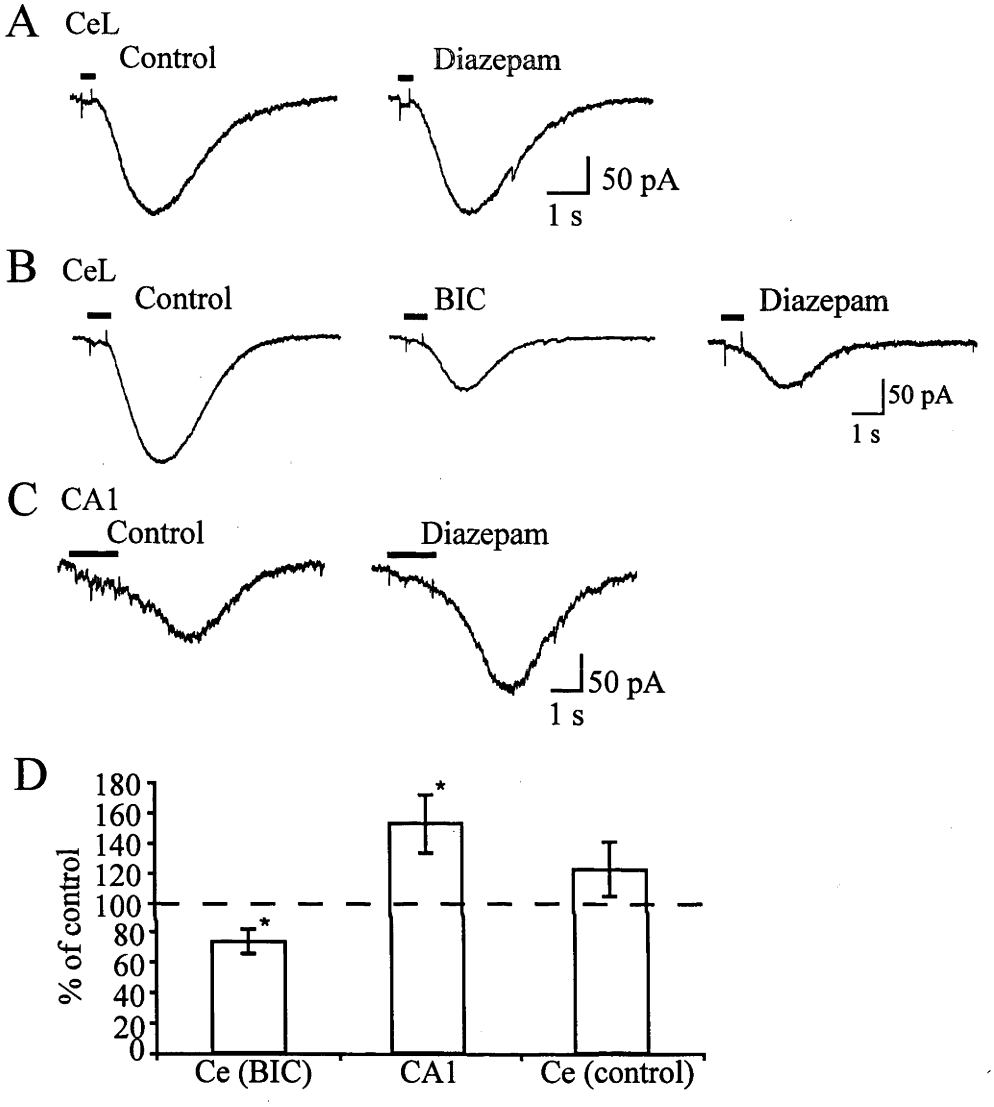

Figure 4.13. Effect of diazepam on GABA iontophoresis responses.
A. Diazepam (10 μM) effect on the control GABA response current of a CeL neurone (average of six responses). GABA iontophoresis duration is indicated by bars. B. Diazepam (10 μM) applied to the GABA iontophoresis response remaining after block of the $GABA_A$ component by 10 μM bicuculline (average of six responses shown). C. Diazepam effect on the $GABA_A$ mediated response of a hippocampal CA1 neurone (average of six responses shown). D. Average effect of diazepam on response amplitude for Ce neurone responses recorded in bicuculline (10 μM), CA1 neurone responses and Ce neurone responses in control conditions ($n = 5$ for each experiment, $p < 0.05$).

the $GABA_A$ type receptors. This indicates that like the $GABA_A$ receptors of the CA1 neurones, $GABA_A$ receptors of the CeL are positively modulated by diazepam.

To confirm that the inhibitory effect of diazepam was also seen at synaptic responses mediated by the bicuculline insensitive GABA receptor, an IPSC was evoked by lateral stimulation in the presence of bicuculline (10 μM). Diazepam (1 and 10 μM) was applied (Figure 4.14.A) and, as seen for the iontophoretic responses previously, the bicuculline insensitive IPSC were reduced by diazepam. The average block of the bicuculline resistant IPSC amplitude was 28.2 ± 7.0% by 1 μM diazepam ($n = 5$) and 42.2 ± 5.0% by 10 μM ($p < 0.05$, $n = 5$) (Figure 4.14.C). The 1/2 peak width of the bicuculline resistant IPSC remained unaffected after diazepam block (10.3 ± 8.7% decrease in 1 μM, 4.4 ± 4.3% increase in 10 μM, $n = 5$ for each concentration (Figure 4.14.C)). In contrast to these results, control $GABA_A$ mediated IPSC recorded from pyramidal neurones from CA1 were not reduced in amplitude (34.0 ± 15.7% increase in 1 μM diazepam ($n = 5$), 34.0 ± 14.8% increase in 10 μM diazepam ($n = 5$)) but were increased in 1/2 peak width in 1 μM and 10 μM diazepam (16.6 ± 12.0% at 1 μM ($n = 5$), and 32.2 ± 11.9% ($p < 0.05$, $n = 5$), Figure 4.14.B,C).

A more potent 1,4-benzodiazepine, flurazepam, also reduced the amplitude of the CeL IPSC recorded in bicuculline (Figure 4.15.A), with the average block by flurazepam (1 μm) of 36.6 ± 5.0% ($p < 0.05$, $n = 9$, Figure 4.15.C). Again only a small insignificant decrease in the 1/2 peak width (-6.5 ± 5.8%) accompanied this effect (Figure 4.15.C). The effect of flurazepam on the $GABA_A$ component of the CeL IPSC was consistent with the effect of these agents on $GABA_A$ receptors (as shown by the diazepam effect in CA1 IPSC previously). The $GABA_A$ mediated IPSC recorded in TPMPA (60 μM) being unaffected in amplitude (4.7 ± 5.5%, $n = 5$) but significantly lengthened in 1/2 peak width (21.2 ± 3.0%, $p < 0.01$, $n = 5$) (Figure 4.15.B,C).

Although the reversal of the effect of a benzodiazepine negative allosteric modulator to a positive modulator has been shown for certain combinations of $GABA_A$ receptor subunits (Puia *et al.*, 1991), the reversal of the classical positive

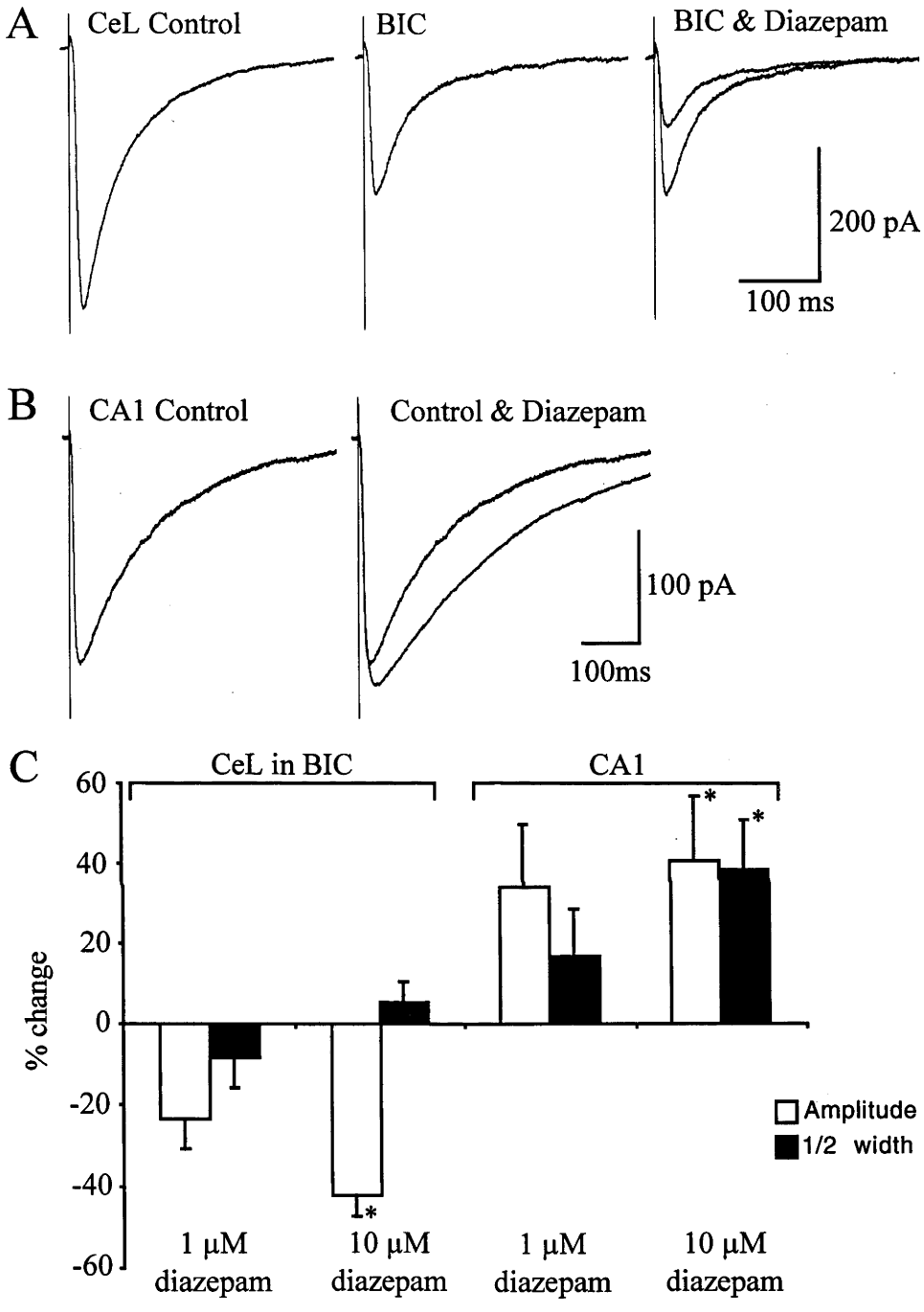

Figure 4.14. Diazepam blocks IPSCs mediated by bicuculline insensitive GABA receptors.

A. Laterally evoked CeL IPSCs recorded in control (10 μM CNQX, 30 μM D-APV), bicuculline (10 μM) and diazepam (10 μM) with average IPSC in bicuculline overlaid. B. CA1 IPSC recorded in control (2 mM kynurenic acid), and diazepam (10 μM) with control IPSC overlaid. C. Effects of 1 and 10 μM diazepam on amplitude and 1/2 width of bicuculline resistant IPSCs recorded in CeL compared to $GABA_A$ mediated IPSC recorded in CA1. ($p < 0.05$, $n = 5$ for each experiment) (Average IPSCs shown).

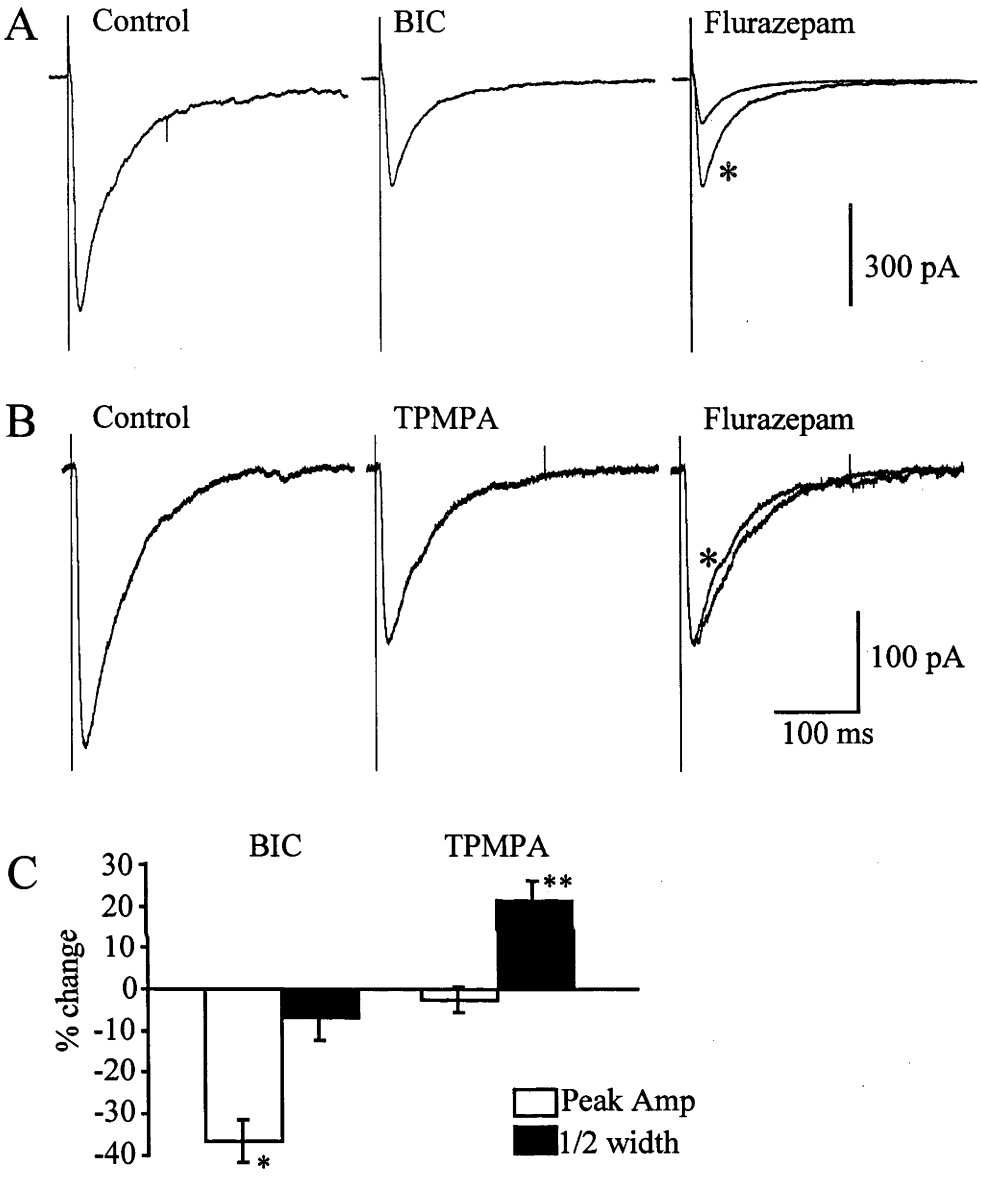

Figure 4.15. Flurazepam reduces the amplitude of the bicuculline resistant IPSCs and increases the decay of the bicuculline sensitive IPSCs.
A. IPSCs recorded in control (10 μM CNQX, 30 μM D-APV), bicuculline (10 μM) and flurazepam (1 μM) (with BIC overlaid - indicated by *).
B. IPSCs recorded in control (2 mM kynurenic acid), TPMPA (60 μM) and flurazepam (1 μM) (with TPMPA overlaid - indicated by *).
C. Average effect of flurazepam on IPSC amplitude and 1/2 peak width for IPSCs recorded in CeL in bicuculline (10 μM) ($p < 0.05$, $n = 9$) and TPMPA (60 μM) ($p < 0.01$, $n = 5$). (Average IPSCs shown.)

modulators, such as diazepam and flurazepam, has not. To establish whether this effect was occuring at a specific benzodiazepine binding site, the benzodiazepine site antagonist Ro 15-1788 was used. This compound is a high affinity ligand for the benzodiazepine site, while not functional as an allosteric modulator of the receptor. It has been shown to be able to act as a competitive inhibitor for the benzodiazepine site, and antagonise the binding of other benzodiazepines (Sieghart, 1995). Application of Ro 15-1788 (1 μM) had no effect on the bicuculline resistant IPSC alone (1.3 ± 2.9% change in amplitude, n = 3), but pre-applying Ro 15-1788 blocked the negative modulation effect of flurazepam on the bicuculline insensitive component of the CeL IPSC (Figure 4.16.A) from the 36.6 ± 5.0% decrease seen previously, to an insignificant 1.9 ± 5.4% increase (n = 4) (Figure 4.16.B). The effect of diazepam was similarly reduced from 42.2 ± 5.0% in bicuculline only, to 2.1 ± 1.5% (n = 3) in bicuculline and Ro 15-1788 (Figure 4.16.B). On washout of the Ro 15-1788 (Figure 4.16.A), the effect of flurazepam was to again reduce the amplitude of the bicuculline resistant IPSC (n = 1).

4.6.5. The effect of propofol on the bicuculline resistant IPSC

Although $GABA_C$ receptors are modulated by some anesthetics, these effects are distinct from the modulation of $GABA_A$ receptors in that those agents negatively modulate the $GABA_C$ response. Other agents (propofol included) were found to have no effect at $GABA_C$ receptors but positively modulate $GABA_A$ responses. The effect of propofol on the bicuculline insensitive IPSC thus may also be a distinguishing pharmacological property. Applying propofol (10 μM) to the laterally stimulated bicuculline resistant IPSC (Figure 4.17.A) resulted in no significant change to either IPSC amplitude or 1/2 peak width (0.53 ± 8.1% increase in amplitude, and 6.9 ± 12.9% decrease in 1/2 width, n = 3, Figure 4.17.C). The 1/2 width of $GABA_A$ mediated IPSC recorded in hippocampal pyramidal neurones of the CA1 region (Figure 4.17.B) were increased by the same concentration of propofol by 136.9 ± 21.8% ($p < 0.05$, n = 3), while the amplitude of these responses was unchanged (0.3 ± 13.9% of control, n = 3) (Figure 4.17.C).

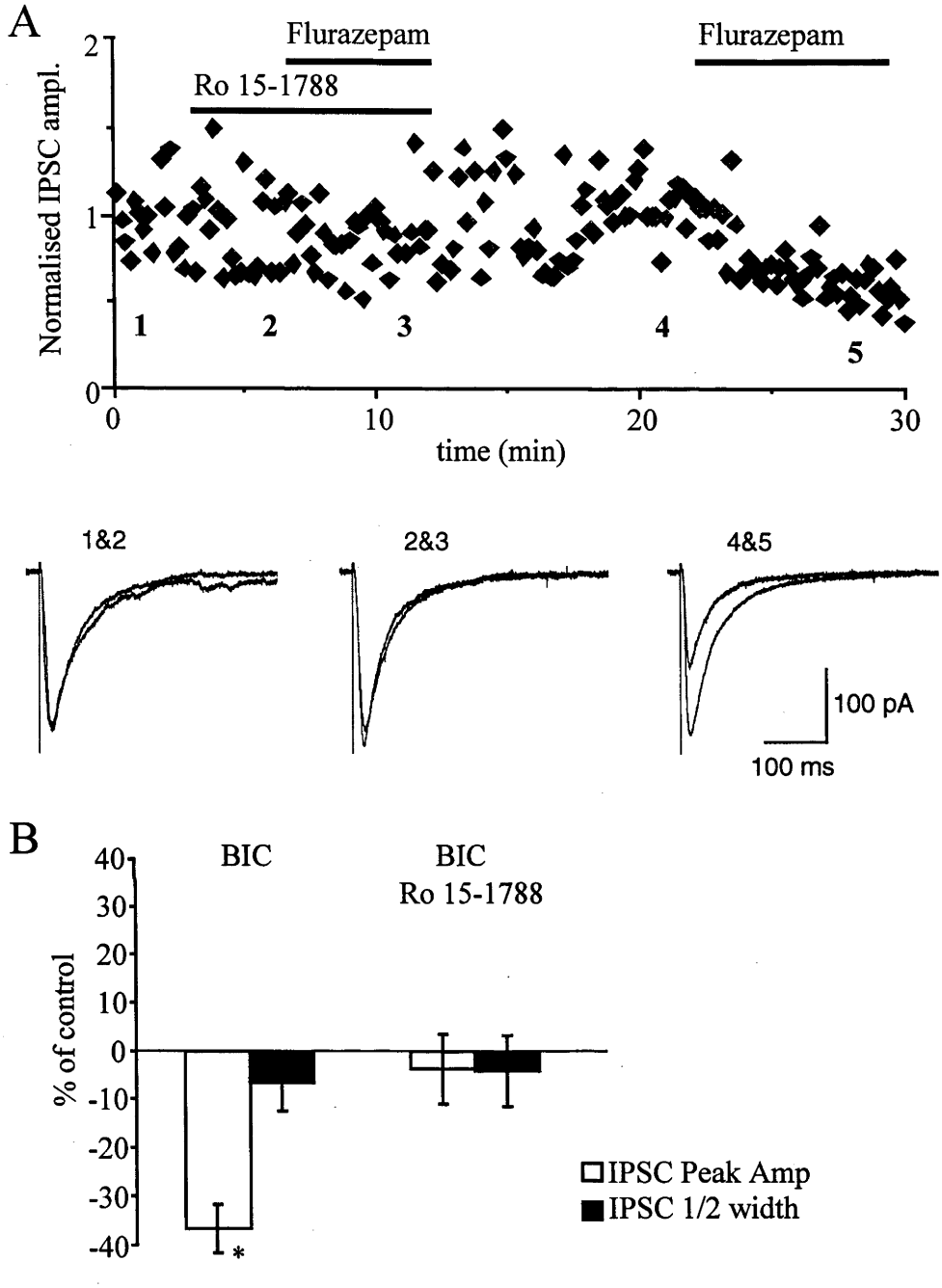

Figure 4.16. Ro 15-1788 inhibits the flurazepam block of bicuculline insensitive IPSCs.

A. Plot of normalised IPSCs recorded in kynurenic acid and bicuculline (2 mM and 10 μM respectively) in control (1), Ro 15-1788 (1 μM) (2), Ro 15-1788 and flurazepam (1 μM) (3), after washout to control (4) and in flurazepam (1 μM) (5). B. Average effect of flurazepam on laterally evoked CeL IPSCs in bicuculline ($p < 0.05$, $n = 9$) and in bicuculline and Ro 15-1788 ($n = 4$). (IPSCs shown are averages of six individual IPSCs.)

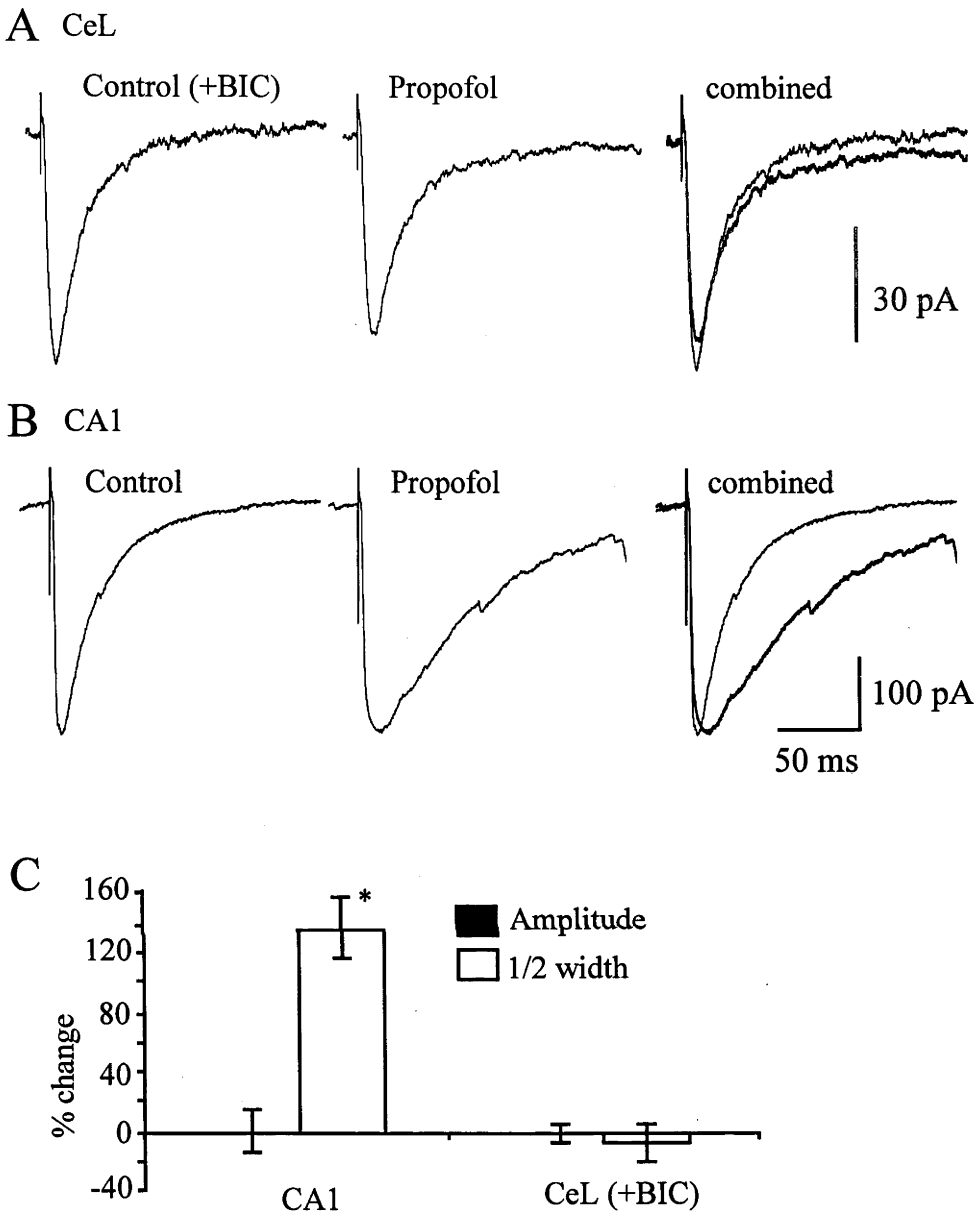

Figure 4.17. CeL IPSCs mediated by bicuculline resistant GABA receptors are insensitive to propofol.

A. Laterally stimulated CeL IPSCs in bicuculline (10 μM), bicuculline and propofol (10 μM) and these responses overlaid. B. $GABA_A$ mediated IPSCs recorded in a pyramidal hippocampal neurone in control, propofol (10 μM), and these responses overlaid. C. Average effect of propofol on CA1 IPSCs compared to CeL IPSCs recorded in bicuculline ($p < 0.05$, $n = 3$ for each location). (IPSCs shown are averages of six individual IPSCs.)

4.6.6. The effect of pentobarbitone on the bicuculline resistant IPSC

Like the benzodiazepines, barbiturates have also been found to be specific modulators of $GABA_A$ receptors. The effect of the barbiturate pentobarbitone was also examined on the bicuculline resistant GABA response.

The bicuculline resistant component of the CeL IPSC was positively modulated by the addition of a low concentration of pentobarbitone (25 μM) to the extracellular solution (Figure 4.18.A), however the extent of this modulation was less than that shown by the control $GABA_A$ mediated IPSC recorded in CA1 (Figure 4.18.B). Whereas the CA1 IPSC was increased in 1/2 width by 166 ± 29.5% ($p < 0.01$, $n = 4$), the bicuculline resistant component of the CeL IPSC was increased by only 32.7 ± 11.8% ($p < 0.05$, $n = 3$). Furthermore, the amplitude of the control CA1 response was not changed by pentobarbitone (2.6 ± 24.2% increase, $n = 4$), but the CeL IPSC was reduced in amplitude by 21.5 ± 5.7% ($p < 0.05$, $n = 3$).

Addition of higher concentrations of pentobarbitone (100 μM), sufficient to directly activate GABA receptors, did so on both CeL neurones in the presence of bicuculline and CA1 neurones. This phenomenon is believed to occur at a site distinct from the site of action for the modulation effect, and can occur even in the absence of the agonist GABA (Sieghart, 1995). As both $GABA_A$ and $GABA_C$-like receptors are directly activated by 100 μM pentobarbitone (even in the presence of bicuculline) the direct activation current in CeL neurones could have resulted from activation of either or both receptor types.

4.6.7. $GABA_B$ responses were not seen in CeL neurones

Bath application of the selective $GABA_B$ antagonist baclofen produced no change in membrane resistance, nor did it activate any significant potassium currents (n=3). Isolated IPSC recorded using potassium methylsulfate based internal solution show no slow IPSC components similar to those previously recorded postsynaptically in hippocampus and nucleus reticularis thalami (Ling & Bernardo, 1994; Ulrich & Huguenard, 1996). These results suggest that $GABA_B$ receptors are not present at

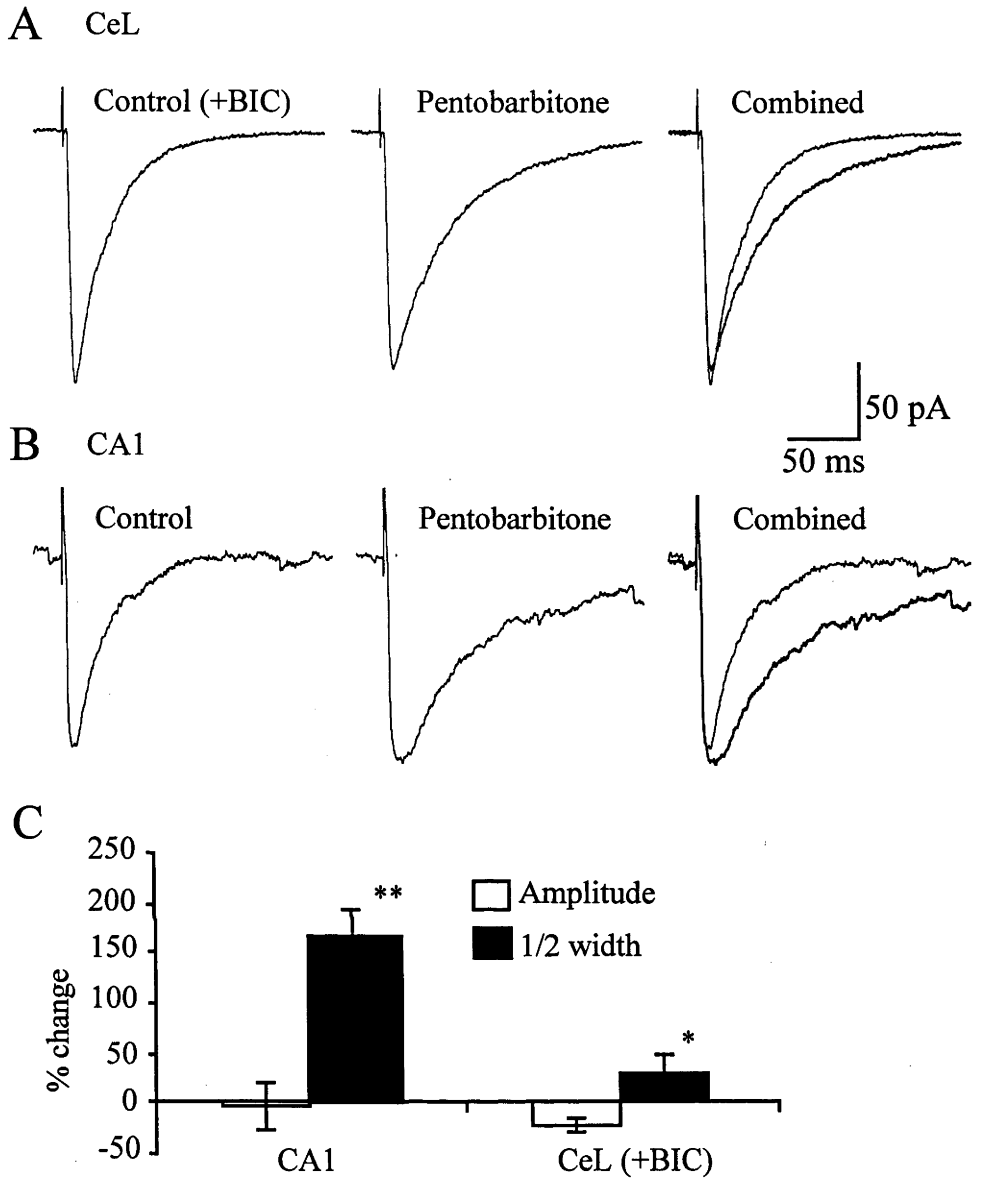

Figure 4.18. CeL IPSCs mediated by bicuculline resistant GABA receptors are show less modulation by pentobarbitone.
A. Laterally stimulated CeL IPSCs in bicuculline (10 μM), bicuculline and pentobarbitone (25 μM) and these responses overlaid. B. $GABA_A$ mediated IPSCs recorded in pyramidal hippocampal neurone in control, pentobarbitone (25 μM), and these responses overlaid. C. Average effect of pentobarbitone on CA1 IPSCs compared to CeL IPSCs recorded in bicuculline ($p < 0.01$, $n = 4$ for CA1, $p < 0.05$, $n = 3$ for CeL). (IPSCs shown are averages of six individual IPSC.)

high density on these neurones, however the recordings were made at room temperature which may affect the size of the $GABA_B$ response, rendering it undetectable.

4.7. DISCUSSION

4.7.1. Two types of GABA receptors are expressed by ceL neurones.

Although the neurones of the Ce express both glycine and GABA receptors, the fast inhibitory transmission at CeL synapses is mediated by GABA receptors only. Two ionotropic GABA receptors are found on Ce neurones, one is typical of the $GABA_A$ type receptor, having high affinity for the competitive antagonists bicuculline and SR95531. This receptor mediates approximately 60% of the IPSC resultant from the activation of lateral monosynaptic inhibitory inputs. The other GABA receptor contributing to the IPSC is less sensitive to the antagonists SR95531, bicuculline, and the antagonist picrotoxin than $GABA_A$ receptors expressed by hippocampal pyramidal neurones, and sensitive to the $GABA_C$ antagonist TPMPA. Furthermore, the bicuculline insensitive IPSC mediated by these receptors was blocked by the 1,4-benzodiazepines diazepam and flurazepam, unaffected by the anaesthetic propofol and less sensitive to the barbiturate pentobarbitone than hippocampal $GABA_A$ responses.

4.7.2. $GABA_A$ or $GABA_C$?

The aim of the preceding experiments was to establish the nature of bicuculline resistant GABA receptors, in terms of the pharmacological profile of the receptor. The drugs used have typical effects on expressed $GABA_A$ and $GABA_C$ receptors. The pharmacological profile exhibited by the bicuculline resistant GABA

receptors was not consistent with either of these receptors however, and arguments can be made for this receptor having properties normally associated with both.

The low bicuculline affinity receptors were less sensitive to picrotoxin and SR95531 than $GABA_A$ receptors and sensitive to the specific $GABA_C$ antagonist TPMPA. These results are consistent with $GABA_C$ type receptors (Qian *et al.*, 1998). The bicuculline resistant receptor was also insensitive to propofol, which is also consistent with the reported pharmacology of $GABA_C$ receptors (Mihic & Harris, 1996). However, the fact that this $GABA_C$-like receptor is sensitive at all to bicuculline is at odds with all present data concerning the known homomeric ρ subunit combinations which have been shown to be insensitive to bicuculline in the 100 μM range (Qian *et al.*, 1998). The effects of the 1,4-benzodiazepines and the barbiturate pentobarbitone at these receptors is also inconsistant with the reported insensitivity of $GABA_C$ receptors to these drugs (Bormann & Feigenspan, 1995; Johnston, 1996; Shimada *et al.*, 1992). Indeed the presence of a binding site for the benzodiazepines indicates the presence of $GABA_A$ α and γ subunits as part of the receptor structure, as these subunits have been shown to form the binding site for these drugs (Macdonald & Olsen, 1994; Sieghart, 1995; Sigel & Buhr, 1997).

If the low bicuculline affinity receptors are composed of $GABA_A$ receptor subunits including an α and a γ type, then this combination would be unique in its modification by classical 1,4-benzodiazepines such as diazepam and flurazepam. Though variability in the efficacy of diazepam (including insensitivity) has been shown for a range of alternate α and γ subunit compositions (Costa & Guidotti, 1996; Puia *et al.*, 1992; Puia *et al.*, 1991) none of the α,β and γ combinations examined have shown negative allosteric modulation by diazepam. The only classical benzodiazepine known to act as a negative modulator is 4'-chlorodiazepam, though this action is thought to occur at a different site than the positive modulation as it is not antagonised by the specific site antagonist flumazenil (Ro 15-1788). The reversal of other positive modulators which act at the benzodiazepine site has been reported for the imidazopyridines alpidem and zolpidem, at recombinant GABA receptors containing α_3, β_1 and γ_1 and α_2,β_1 and γ_1

subunits respectively (Puia *et al.*, 1991). Furthermore, the reversal of the action of negative allosteric modulators which act at the benzodiazepine site (becoming positive modulators) has been demonstrated, with the β-carboline derivatives DMCM and β-CCM acting as positive modulators of recombinant GABA receptors containing α_1, α_2, α_3, and α_5 with β_1 and γ_1 subunits, and α_1 and α_2 with β_1 and γ_1 subunits respectively (Puia *et al.*, 1991). These effects are all coincident with the presence of a γ_1 subunit in the GABA receptor complex. It is feasible in light of these results and the fact that the expression of γ_1 is high in the Ce (Wisden *et al.*, 1992) that the inhibitory effects of the classical benzodiazepines on the receptors in CeL are also the result of these agents binding to GABA receptor which is a combination of known $GABA_A$ subunits (including a γ_1 subunit) which has not been examined for benzodiazepine effect. One such possibility is the ε subunit.

Functional recombinant $GABA_A$ receptors containing the ε subunit expressed in combination with α_1 and α_2 with β_1 and β_3 subunits expressed in HEK-293 cells were found to be insensitive to propofol and less sensitive to pentobarbital than $\alpha_2\beta_1$ receptor complexes (Davies *et al.*, 1997). Furthermore the sensitivity of these receptors to bicuculline and picrotoxin was also less that expected for typical $GABA_A$ responses (73.6 ± 9.3% block by 10 μM bicuculline and 82.5 ± 7.4% block by 10 μM picrotoxin), and these receptor showed slightly higher agonist sensitivity. The different bicuculline and agonist may also indicate some differences in the agonist binding site, which may provide the sensitivity to the constricted GABA analogue and $GABA_C$ receptor antagonist TPMPA. Finally, and perhaps coincidentally, the ε subunit is also highly expressed in the amygdala (Davies *et al.*, 1997).

While the subunit combinations expressed with the ε subunit in the recombinant study by Davies *et al* would not be expected to be modulated by benzodiazepines in the absence of a γ subunit, it is conceivable that alternate combinations of subunits with ε (including the γ_1 subunits both highly expressed in amygdala) may also provide unusual anaesthetic and benzodiazepine pharmacology. Alternately, it is not inconceivable that a previously undescribed variant of a known $GABA_A$ subunit, a novel subunit or one of the ρ type subunits may instead confer this unusual

benzodiazepine pharmacology by being incorporated into a receptor with the α and γ subunits required for forming a benzodiazepine site as has been suggested for recently described bicuculline insensitive GABA responses in rat spinal dorsal horn which also show benzodiazepine sensitivity (Park *et al.*, 1999).

In conclusion, these results also demonstrate the presence of two pharmacologically distinct GABA receptor subtypes on CeL neurones. One type is typical of $GABA_A$ receptors elsewhere in the brain, being blocked by low concentrations of bicuculline and SR95531, and being positively modulated by the 1,4-benzodiazepines diazepam and flurazepam. The other type of receptor has pharmacological properties similar to both $GABA_A$ receptor types – modulation by 1,4-benzodiazepines (albeit negative modulation) and pentobarbitone, and insensitivity to propofol (those containing the ε subunit), and to $GABA_C$ receptor types – insensitivity to propofol and sensitivity to block by TPMPA, and higher doses of picrotoxin and SR95531.

5. THE SYNAPTIC DISTRIBUTION OF TWO GABA RECEPTORS ON CeL NEURONES

5.1. INTRODUCTION

5.1.1. Co-localisation of GABA receptors

Differential synaptic distribution of distinct $GABA_A$ receptor subunits at different synapses on the same neurone has been demonstrated histologically (Fletcher *et al.*, 1998; Gustincich *et al.*, 1999; Nusser *et al.*, 1996) suggesting that compositionally distinct GABA receptors are localised at separate synapses. $GABA_C$ receptors have also been shown histologically to be localised to separate synapses to $GABA_A$ receptors (Fletcher *et al.*, 1998; Koulen *et al.*, 1998). In the previous chapter we described two pharmacologically distinct GABA receptors contributing to synaptic currents on CeL neurones. Both receptors were found to contribute to the large postsynaptic inhibitory currents in response to electrical stimulation of multiple presynaptic fibres. This chapter will address the synaptic co-localisation of these receptors at the individual synapses on CeL neurones by examining the pharmacological effects of bicuculline and TPMPA at individual synapses on CeL neurones.

5.1.2. Miniature spontaneous IPSC

In the presence of the sodium channel blocker tetrodotoxin, neurones are unable to fire action potentials (Takahashi, 1984). Under these conditions, small spontaneous synaptic responses can be recorded as vesicles of transmitter are spontaneously released from presynaptic terminals (Collingridge *et al.*, 1984; Fatt & Katz, 1952; Takahashi, 1984). These spontaneous responses – called miniature postsynaptic currents (mPSCs) or potentials (mPSPs), are believed to be responses from a single postsynaptic site to the release of a single vesicle of neurotransmitter (Edwards *et al.*, 1990; Katz, 1978). As such, spontaneous PSCs and PSPs have been studied

extensively for the information that they reveal about the unitary nature of synaptic transmission such as release probability, quantal size and the kinetics of the post synaptic response.

One of the most useful features about studying mPSC's is that these responses result from the activation of single postsynaptic site. This has been exploited in pharmacological studies to determine the receptors underlying the postsynaptic response (Bekkers & Stevens, 1989; Legendre, 1997; McBain & Dingledine, 1992). Similarly, the effects of bicuculline and TPMPA were examined on the mIPSC population recorded in CeL neurones to determine the contribution of the $GABA_A$ and the bicuculline resistant GABA receptors to postsynaptic currents at individual synapses on these neurones.

5.2. RESULTS

5.2.1. The effects of BIC and TPMPA on CeL mIPSCs

To determine whether both the $GABA_A$ and the bicuculline resistant GABA receptors contribute to synaptic currents at individual synapses on CeL neurones, we examined the effects of the antagonists bicuculline and TPMPA on the miniature spontaneous IPSCs (mIPSCs) recorded in tetrodotoxin (TTX) and kynurenic acid to block spontaneous glutamatergic responses. Membrane current was filtered at 5 kHz and sampled at 10 kHz in consecutive 3 second sweeps. The sweeps were then digitally refiltered at 1 kHz, and individual events were detected using a variable amplitude template. The template function was a sum of a rising exponential function (with the rise time set to 1ms) and a decay exponential (with the decay time constant set to 12 ms). Events were recorded with a 10 ms baseline and rejected if another event occurred within 60 ms of the onset of the rising phase. Experiments were performed at Vm –60 mV, using CsCl based internal solution.

Two types of miniature PSCs were collected in control conditions at Vm -60 (Figure 5.1.A,B,C), which could be disinguished by rise and decay kinetics. The first type was fast rising and fast decaying mPSC with similar rise time (less than 1 ms) and decay time (less than 3 ms) to previously reported AMPA mediated EPSC (Bekkers & Stevens, 1996; Jonas *et al.*, 1993). These fast events were not present when recording in either CNQX or kynurenic acid (Figure 5.1.D), confirming that these events were mediated by glutamate receptors. The second type of events recorded in control conditions (5.1.C) were slower in rise time and decay and were not affected by kynurenic acid (5.1.D). Furthermore, the slow events were outward currents at depolarised membrane potentials (-30 mV, 0 mV - Figure 5.2.B,C respectively) and inward at hyperpolarised potemtials (-70 mV - Figure 5.2.A), indicating that they were chloride mediated mIPSCs. The fast decaying events were inward at negative membrane potentials, reversing at Vm 0 (Figure 5.2.C) and were thus mEPSCs.

The slow decaying mIPSCs, isolated in kynurenic acid or CNQX/APV (Figure 5.3.A,B), had an average 10 – 90 % rise time of 1.5 ± 0.1 ms (n = 14 cells), and decay usually best fit by two exponentials with time constants of 14.5 ± 0.9 ms for the fast decay phase and 57.9 ± 7.2 ms for the slow. These rise and decay times are similar to previously reported GABAergic mIPSCs recorded under similar conditions using similar techniques (Ropert *et al.*, 1990). Applying picrotoxin (100 μM), or bicuculline (10 μM) and TPMPA (100 μM) to these events in kynurenic acid blocked all events (Figure 5.3.C), indicating that these events were mediated by GABA receptors as seen previously for the evoked IPSC.

To determine the contribution of $GABA_A$ receptors to the mIPSCs, bicuculline was applied at 3 μM. This concentration was at the top of the first phase of the bicuculline dose response curve (Figure 4.4.), and according to the calculated IC_{50} would block more that 94 % of the $GABA_A$ receptor mediated current but only 10 % of the bicuculline insensitive current. This would minimise the low affinity block of the bicuculline resistant component to maximise the amplitude of the bicuculline insensitive GABA events, and thus facilitate their detection. Furthermore, this dose

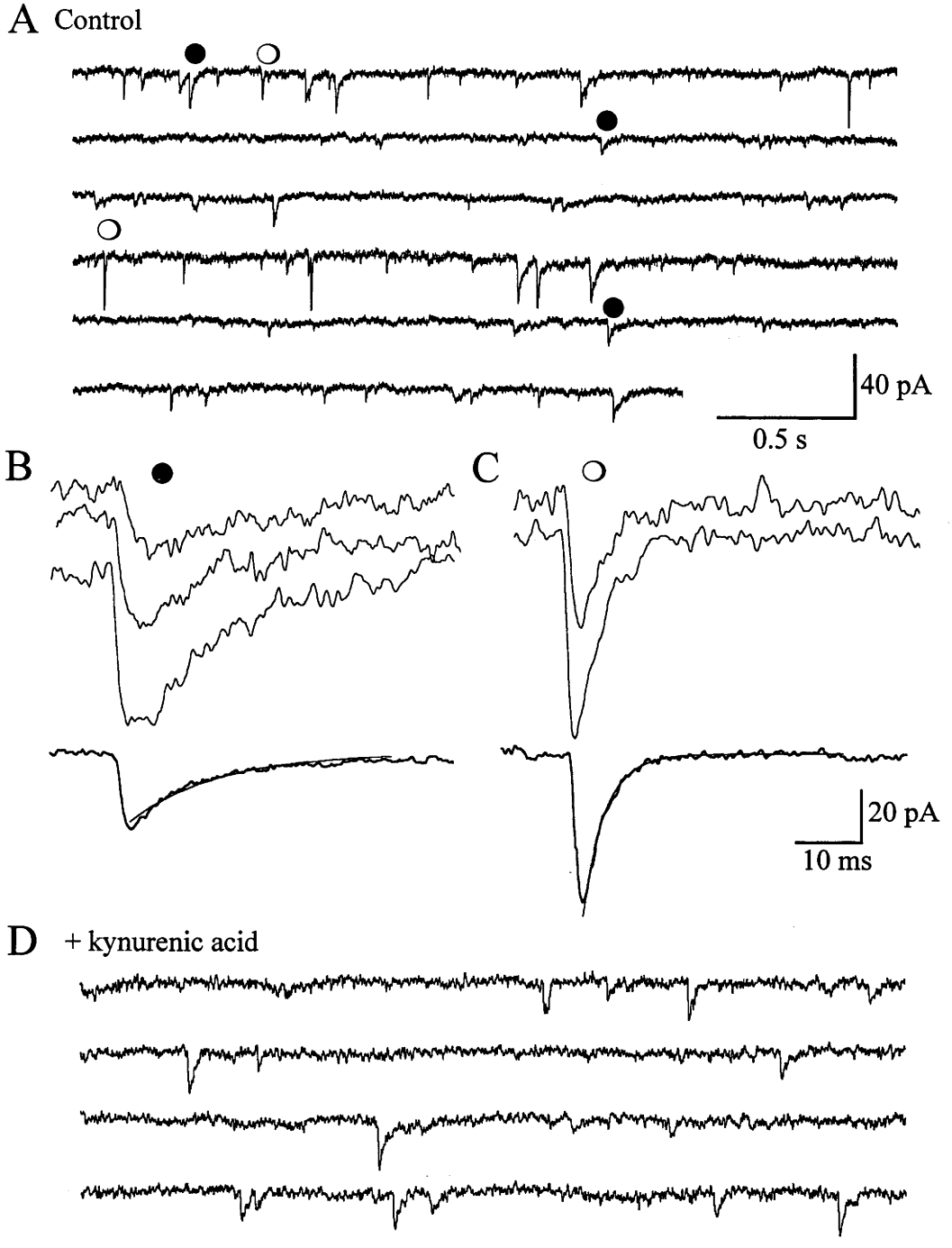

Figure 5.1. CeL mEPSCs are fast rising and decaying.
A. Membrane current recordings showing spontaneous mPSCs in control recording conditions. Individual slow (● in A) and fast (○ in A.) decaying mPSCs (B. and C. respectively), and the average of these events (heavy line under) fit with a single exponential (light line overlaid). The decay constant was 12.9 ms for the slow decaying mPSC average and 3.4 ms for the fast decaying average. D. Membrane current recordings in 2 mM kynurenic acid with only slow decaying events evident .

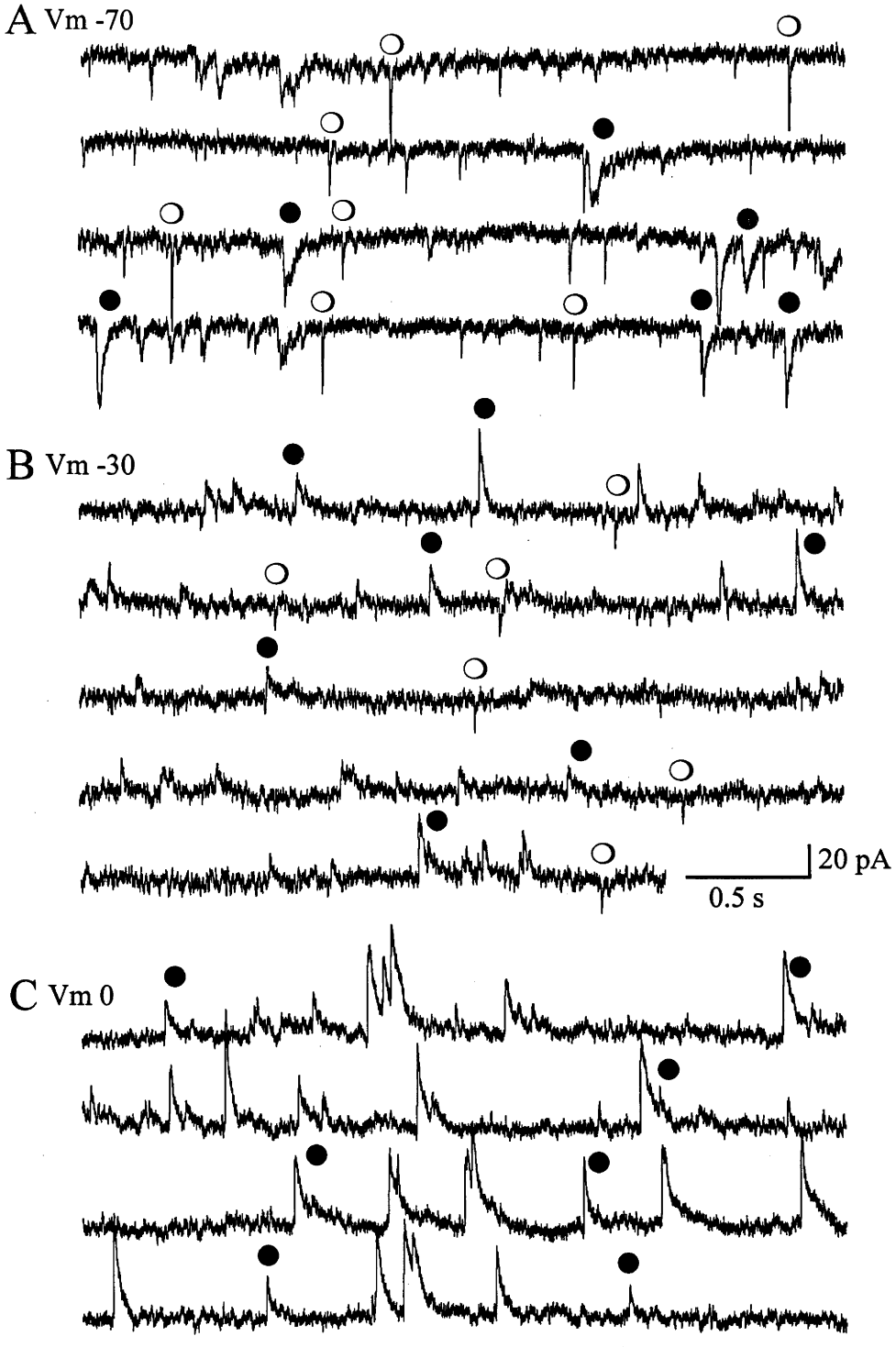

Figure 5.2. Slow mPSCs reverse with membrane depolarisation.
Membrane current recordings in TTX, at membrane potentials -70 mV (A), -30 mV (B) and 0 mV (C). Note the presence of inward fast decaying events (○) in A and B but not C, and the inward slow decaying events (●) in A, which are outward in B and C.

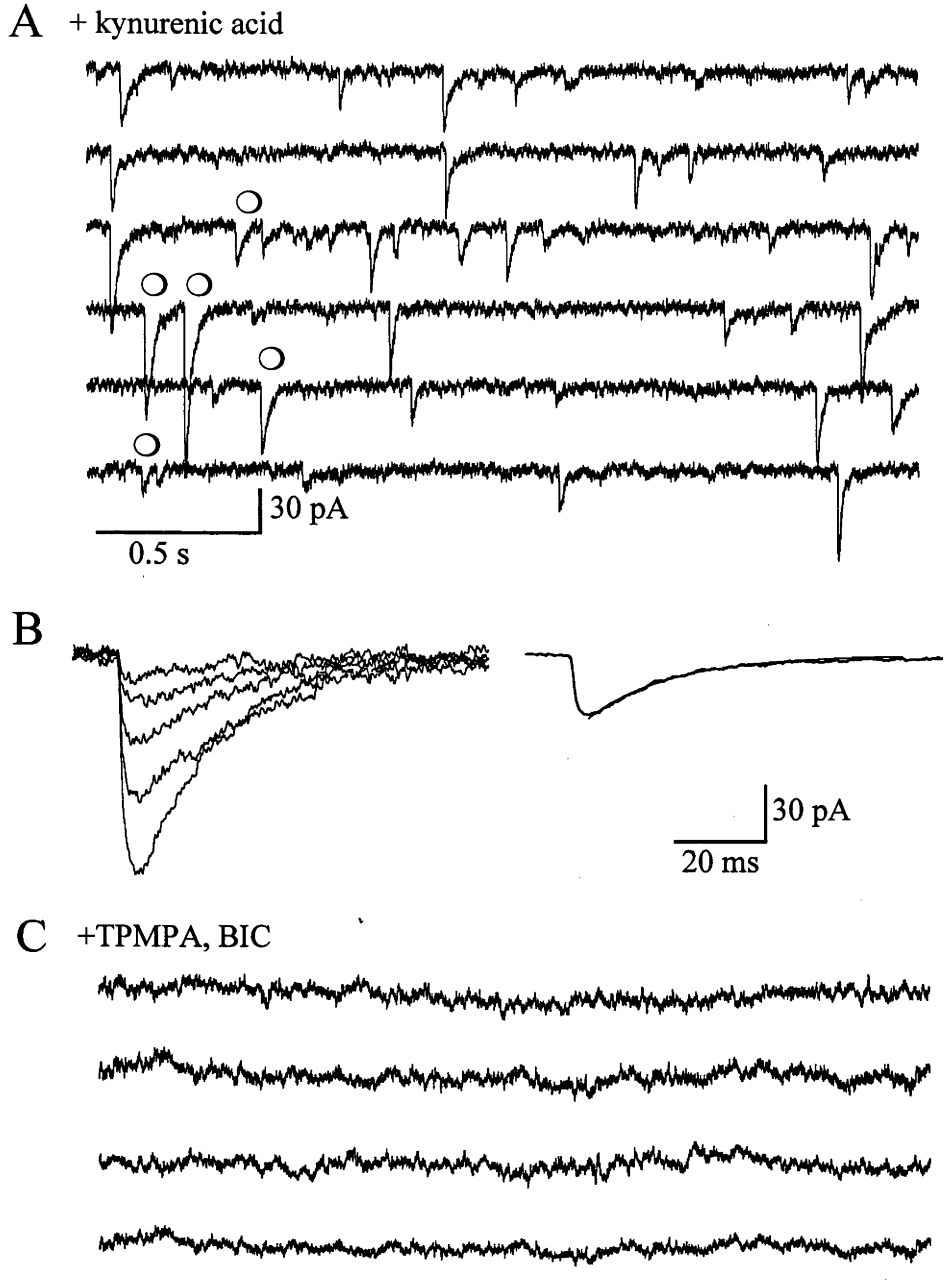

Figure 5.3. CeL mIPSCs are blocked by bicuculline and TPMPA.
A. Membrane current recorded in TTX (0.5 μM) and kynurenic acid (2 mM) showing spontaneous mIPSCs. CsCl based internal solution was used and recordings were made at vm -60. B. Individual events captured from A. (indicated by ○) and the average of all events captured from A. (heavy line at right) fit with a single exponential with time constant 17.8 ms (light line overlaid). C. Membrane current recordings made in TTX, kynurenic acid, bicuculline (10 μM) and TPMPA (100 μM).

is greater than that used previously to block $GABA_A$ mediated mIPSCs in hippocampus (Edwards *et al.*, 1990).

Spontaneous mIPSCs were recorded in the presence of kynurenic acid and bicuculline and TPMPA were applied (Figure 5.4.A,C). After application of either drug, mIPSCs of similar rise time and decay time were recorded, however the amplitude of individual events was smaller than those seen in control (5.4.B,D). Bicuculline reduced the average mIPSC amplitude (Figure 5.5.A), by an average of 71.1 ± 6.2 % ($p < 0.01$, $n = 6$), with the cumulative frequency histogram of peak amplitude was shifted to the left for the entire amplitude range (Figure 5.5.B). Bicuculline however did reduce the mIPSC frequency (Figure 5.5.C, 12.2 ± 3.58 %, $p < 0.05$, $n = 3$). Applying the antagonist TPMPA produced a partial block of the average mIPSC of 37.5 ± 4.1 % ($p < 0.01$, $n = 9$) (Figure 5.6.A). TPMPA was also found to shift the cumulative frequency histogram for mIPSC peak amplitude to the left (Figure 5.6.B), however the TPMPA plot was found to converge with the control plot at high amplitudes. This result may be indicative of insensitivity of some large mIPSC events to TPMPA. TPMPA had no significant effect on the mIPSC frequency (Figure 5.6.C), indicating that in control recording conditions there are no synapses whose responses are mediated solely by the bicuculline resistant GABA receptors.

The slight reduction of mIPSC frequency after bicuculline block has two potential causes. The first is that the reduction of smaller amplitude control mIPSCs by the average block of bicuculline (63.2 %) would render these events too small to be detected from the background noise level of around 2 pA peak to peak. The second possibility was that there were a small number of control events that were completely blocked by bicuculline, thus reducing the frequency of events. This possibility may also be consistent with the suggestion that some larger events being insensitive to TPMPA. It is probable that both of these factors contributed to the reduction in mIPSC frequency, and that an even smaller number of events than those indicated by the decrease in mIPSC frequency are mediated by $GABA_A$ receptors only. To establish the nature of this putative $GABA_A$ mIPSC population, by examining the effect of bicuculline, a larger sample of mIPSCs in control and in

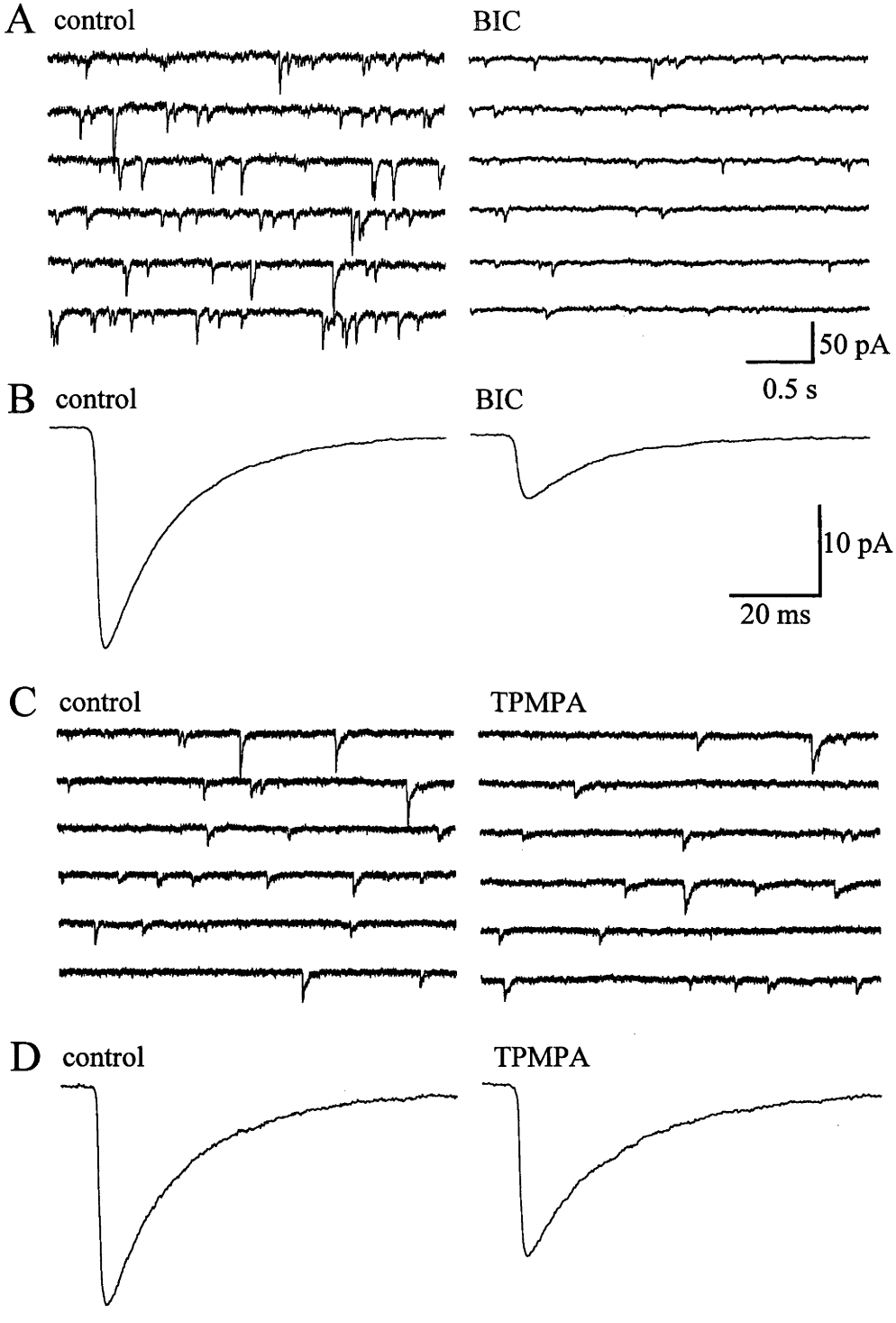

Figure 5.4. Miniature spontaneous IPSCs recorded in bicuculline and TPMPA.

Control mIPSCs were recorded in the presence of 0.5 μM tetrodotoxin, before application of either A. bicuculline (3 μM), or C. TPMPA (60 μM). B. and D. Average mIPSCs from recordings in A. and C. respectively

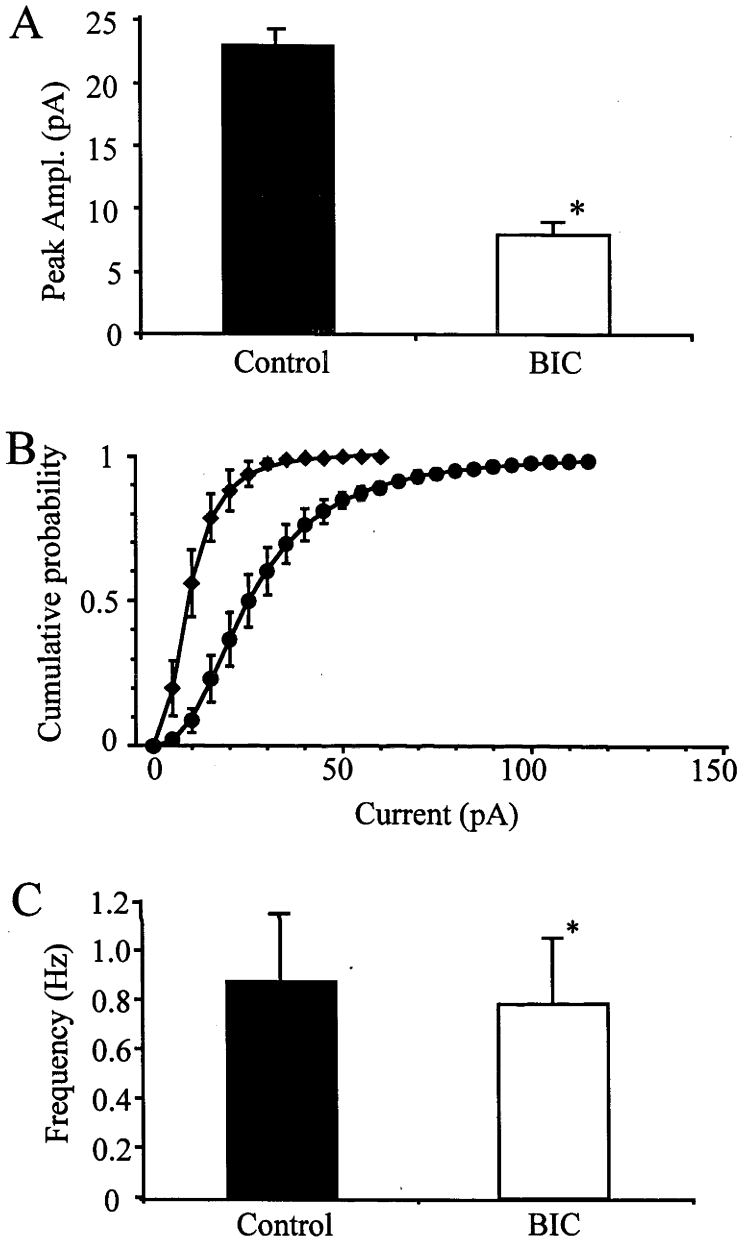

Figure 5.5. Miniature IPSCs are partially blocked by bicuculline.
A. Average mIPSC amplitude for events collected in control and in 3μM bicuculline ($n = 3$, $p < 0.05$). B. Cumulative frequency histogram for mIPSCs collected in three experiments showing a shift to the left when in bicuculline (diamonds). C. Frequency of mIPSCs in control and in bicuculline ($n = 3$, $p < 0.05$).

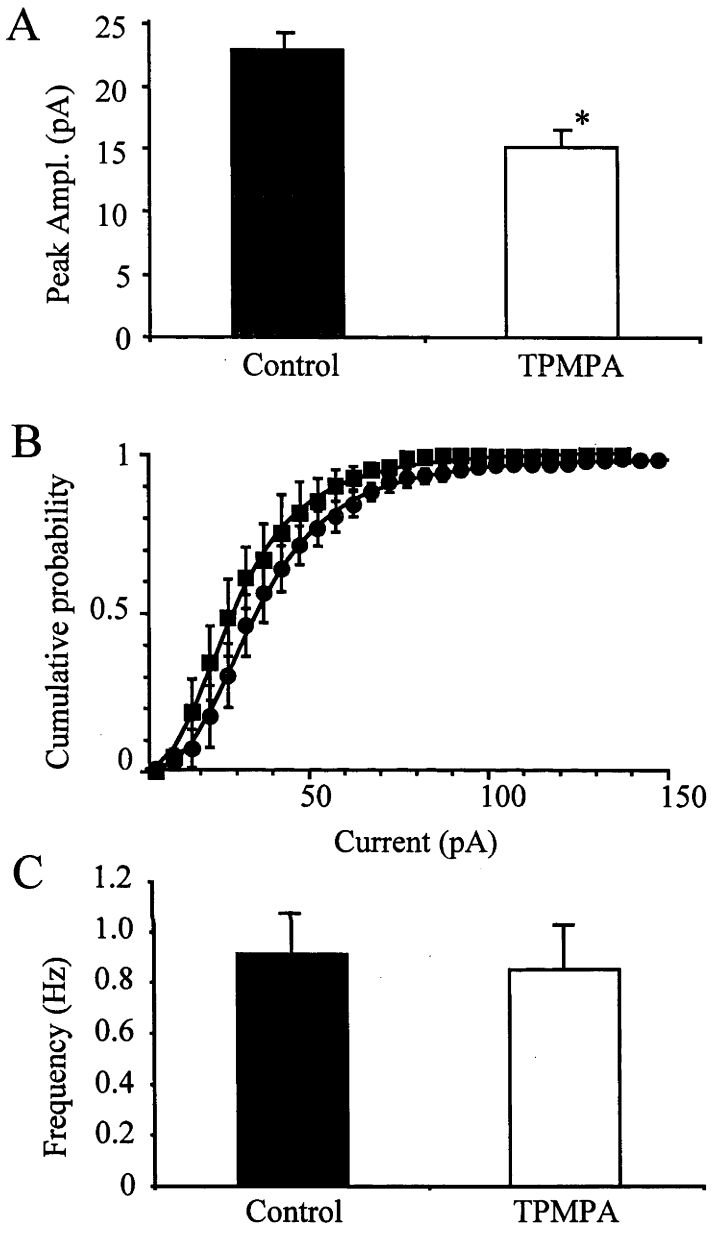

Figure 5.6. Miniature IPSCs are partially blocked by TPMPA.
A. Average mIPSC amplitude for events collected in control and in 60 μM TPMPA ($n = 6$, $p < 0.05$). B. Cumulative frequency histogram for mIPSCs collected in six experiments showing a shift to the left when in TPMPA (squares). C. Frequency of mIPSCs in control and in 60 μM TPMPA ($n = 6$).

bicuculline was required, which would reveal a large enough sample of these events for satisfactory analysis.

5.2.2. Increasing asynchronous release with hypertonic sucrose

To increase the total number of events to allow us to detect a putative $GABA_A$ mediated subpopulation, we applied hypertonic sucrose solution locally to the surface of the slice around the neurone from which we were recording. This method increases the rate of asynchronous release of glutamatergic mEPSC (Bekkers & Stevens, 1989) without affecting the post synaptic response amplitude or kinetic properties (Bekkers & Stevens, 1989; McBain & Dingledine, 1992).

Applying hypertonic sucrose to the surface of the slice consistently produced an increase in mIPSC frequency. However, in 9 out of 12 neurones where the sucrose induced an increase in mIPSC frequency, a population of events which rarely observed in control conditions became prominent (Figure 5.7.A,B). These events were typically greater than 100 pA in amplitude and had faster rise times than the control population. In control recordings where they were detected, the frequency of these events was 0.02 ± 0.002 Hz (n = 6). In the same cells during sucrose applications the frequency of similar amplitude and rise time events was 0.31 ± 0.01 Hz ($n = 6$, $p < 0.01$). The average amplitude of mIPSCs collected when these large events were induced by sucrose application, was 35.4 ± 3.3 pA, compared with 20.4 ± 2.1 pA for control mIPSCs, a 73.5 % increase (Figure 5.7.C, $p < 0.01$, $n = 9$). The 10 – 90 % rise time of 1.1 ± 0.1 ms was also faster than the control average mIPSC 10 – 90 % rise time of 1.5 ± 0.1 ms (a 22.4 % decrease $p < 0.01$, $n = 9$, Figure 5.7.D).

5.2.3. Large fast rising events induced by sucrose are insensitive to TPMPA but blocked by bicuculline

To determine whether the large sucrose induced events were also mediated by both

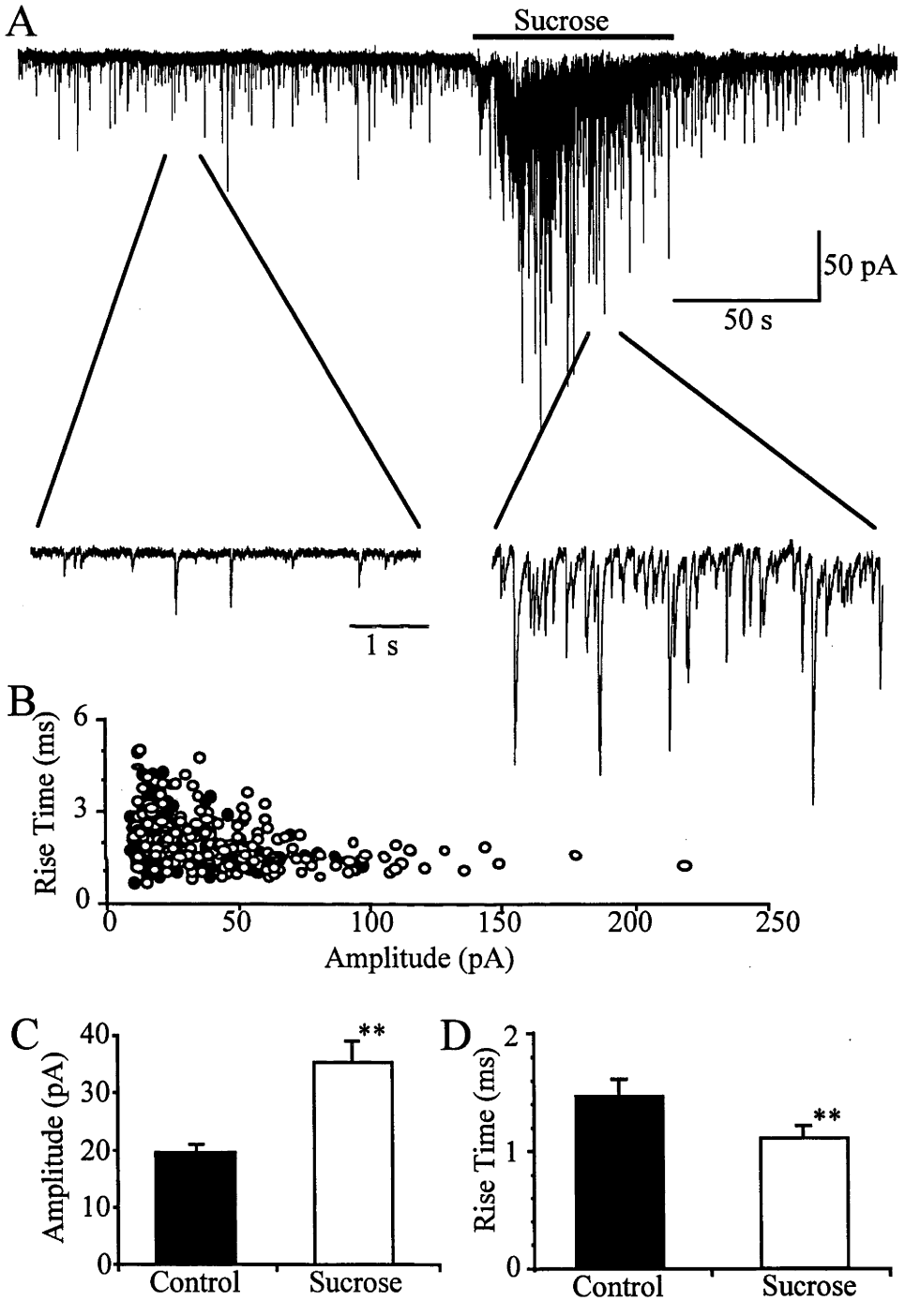

Figure 5.7. Hypertonic sucrose application reveals larger mIPSCs.
A. Hypertonic sucrose solution (500 mM) applied to the surface of the slice induced higher mIPSC frequency and revealed large events seen at very low frequency in control. B. Scatter plot of rise time vs. amplitude showing large amplitude events with fast rise times in sucrose (open circles) where none can be seen in control (closed circles). Average amplitude (C.) and rise time (D.) of mIPSCs in control and sucrose ($p < 0.01$, $n = 9$).

types of GABA receptors, we applied sucrose in the presence of bicuculline and TPMPA. After a control sucrose response of large fast rising mIPSCs was obtained, sucrose was applied in the presence of bicuculline (Figure 5.8.A). Average mIPSC recorded in bicuculline were not significantly different in amplitude (9.0 ± 1.6 pA in control compared to 8.9 ± 2.2 pA in sucrose, 6.0 ± 10.7 % increase, n = 3) or rise time (1.9 ± 0.5 ms in control compared to 2.1 ± 0.5 ms in sucrose, 11.3 ± 5.3 % increase, n = 3) (Figure 5.8.C,D) yet an increase in the frequency of the small amplitude events was seen.

Applying sucrose in the presence of TPMPA (Figure 5.8.B) in contrast, produced both a significant increase in average amplitude, (15.2 ± 2.9 pA in control compared to 29.7 ± 1.7 pA in sucrose, 103.7 ± 25.4 % increase, $p < 0.01$, n = 3) and significant decrease in 10 - 90% rise time (1.9 ± 0.3 ms in control compared to 1.5 ± 0.3 ms in sucrose, 17.1 ± 5.1 % decrease, $p < 0.05$, n = 3) (Figure 5.8.C,D). The average mIPSC recorded in TPMPA, in control and in sucrose, are smaller than of those recorded without TPMPA. This is due to a high proportion of smaller events, in control and sucrose being further reduced by TPMPA. However, the largest 10 % of mIPSCs collected during sucrose application in control and in TPMPA are not significantly different in peak amplitude (control 118.4 ± 7.4 pA vs. TPMPA 113.4 ± 2.1 pA, n = 3) or 10-90 % rise time (control 1.0 ± 0.5 ms vs. TPMPA 1.1 ± 0.3 ms, n = 3) as shown for the averages shown in Figure 5.9. - indicating that this reduction is due to blockade of small events by TPMPA. This also demonstrates that the large events induced by sucrose are not sensitive to TPMPA blockade.

These results indicate that the large, fast rising events induced by sucrose which are not affected by TPMPA and absent in bicuculline are events mediated by bicuculline sensitive $GABA_A$ receptors only. Events of similar amplitude and rise time occur at extremely low frequency or not at all in control recordings without hypertonic sucrose. In cells where they were seen, the average frequency of these events was 0.02 ± 0.002 Hz (n = 6), whereas control mIPSC frequency was 1.62 ± 0.3 Hz (n = 7). Thus two populations of mIPSCs are recorded in CeL neurones, One is a small population of large amplitude fast rising $GABA_A$ mediated events which occur at very low frequency in control, but may be seen at higher frequency when release is

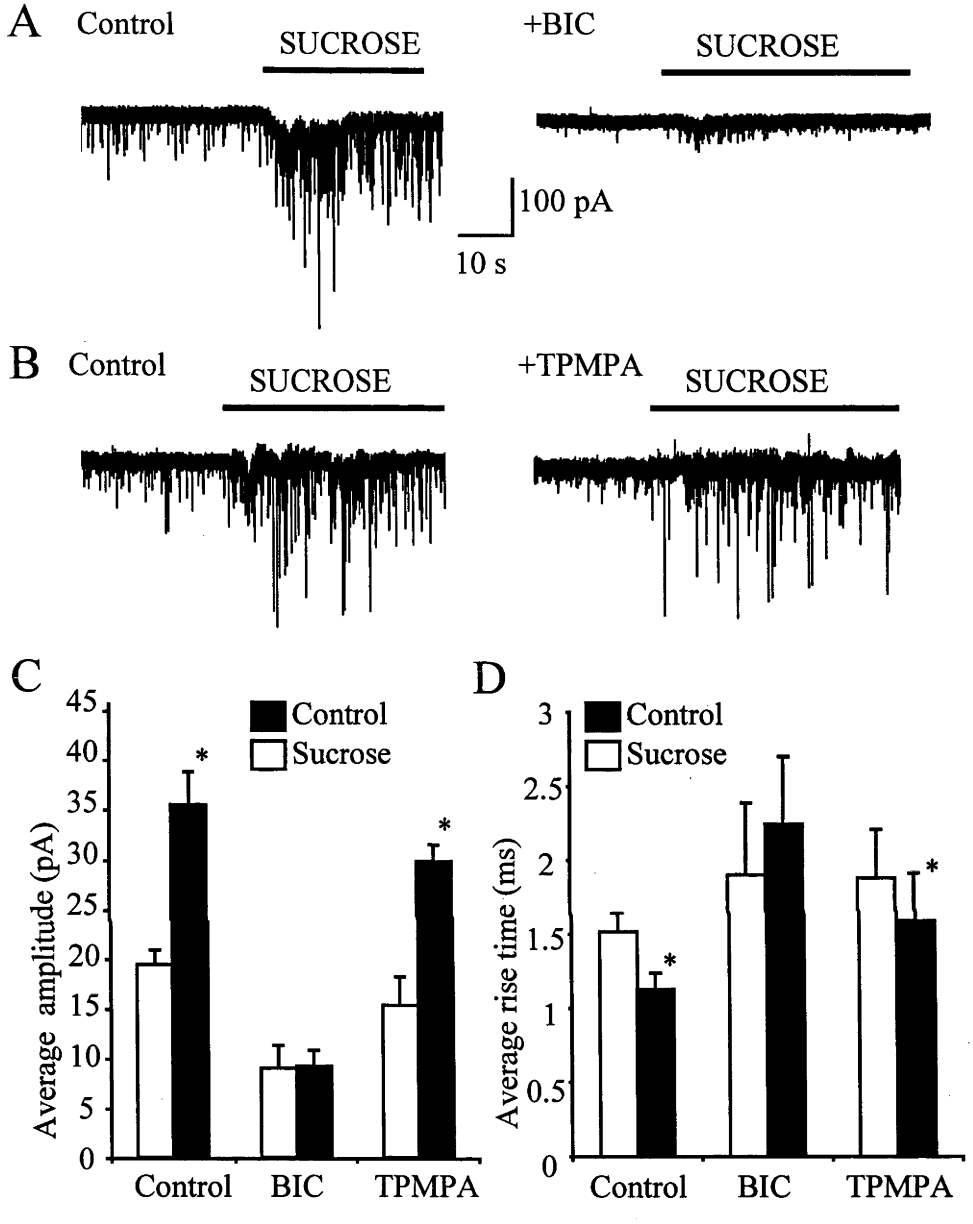

Figure 5.8. Bicuculline blocks large sucrose induced mIPSCs.
A. Application of hypertonic sucrose in control and in bicuculline (10 μM). B. Application of sucrose in control and in 100 μM TPMPA.
C. Average mIPSC amplitude in control, BIC and TPMPA in control and in sucrose ($p < 0.05$, $n = 3$). D. Average mIPSC rise time in control, BIC and TPMPA, in control and in sucrose ($p < 0.05$, $n = 3$).

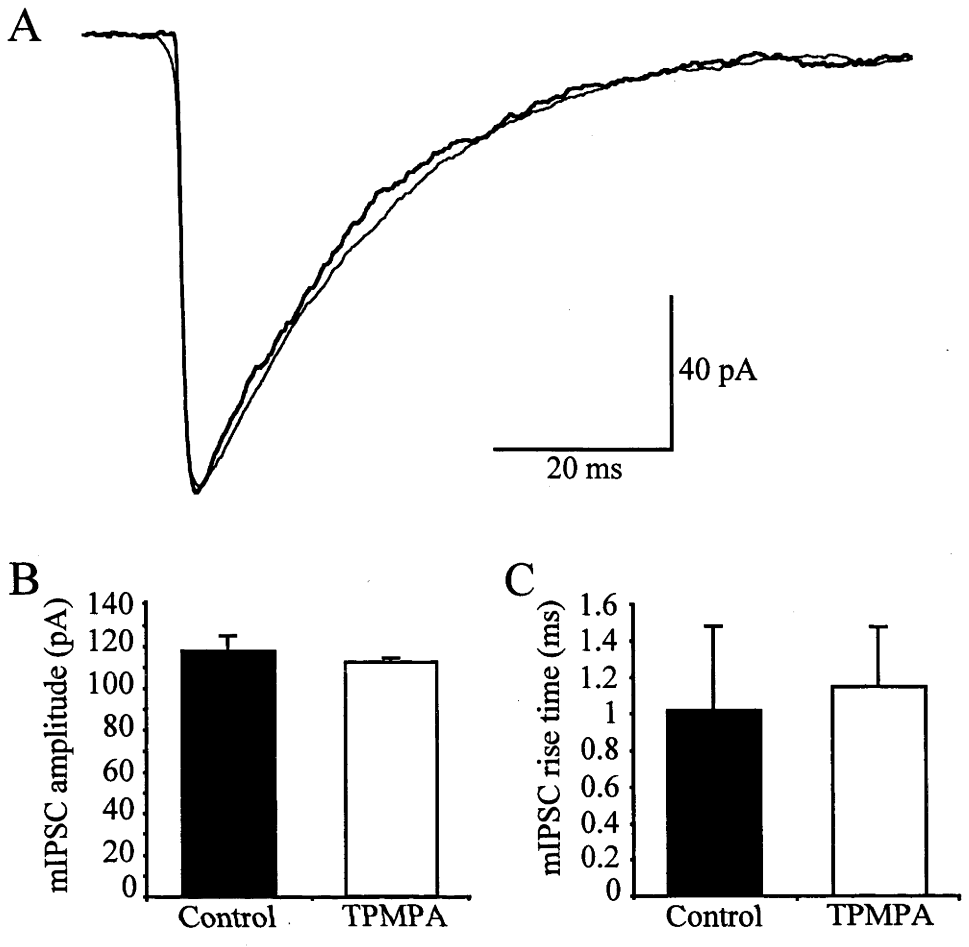

Figure 5.9. Large sucrose induced mIPSCs are TPMPA insensitive.
A. Average of largest 10% of mIPSCs collected during sucrose appliation in control (thin line) and in 100 μM TPMPA (thick line), from experiment shown in Figure 6.5. Average mIPSC amplitude (B.) and rise time (A.) for largest 10% of responses induced by hypertonic sucrose in control and TPMPA, (n = 3 cells).

increased using hypertonic sucrose. The second constitutes the vast majority of events occurring in control recording conditions, and are slower rising smaller amplitude mIPSCs which are mediated by both GABA receptors.

5.2.4. Pure $GABA_A$ synapses are located on the somata of CeL neurones

The fact that the pure $GABA_A$ mediated mIPSCs recorded in hypertonic sucrose are significantly faster rising than the control population of mIPSCs, may be indicative of the localisation of these synapses closer to the soma of the neurone, as has been demonstrated for mEPSCs (Bekkers & Stevens, 1996; Williams & Johnston, 1991). To establish the nature of mIPSCs at somatic and dendritic synapses, hypertonic sucrose solution was applied focally under IR visualisation of the cells to the soma or to dendrites 50 – 100 μM from the soma. In three cases, lucifer yellow was included in the recording pipette and in the sucrose solution and fluorescence was recorded to visualise the neurones and their dendrites, and to indicate the extent of sucrose spread.

Focal application of sucrose to the soma of CeL neurones (Figure 5.10.B) induced a high frequency burst of spontaneous mIPSCs (Figure 5.10.A), which were predominantly large in amplitude and fast rising (n = 3) (Figure 5.10.C). The average mIPSC collected during sucrose application was 327.9 % larger than in control (80.9 pA compared to 19.3 pA in control) and 36.4 % faster in 10 – 90 % rise time (0.7 ms compared to 1.1 ms in control). Histogram plots of events collected in control and sucrose show predominantly small amplitude events in control conditions, but larger events during sucrose application (Figure 5.10.E). Similarly, the distribution of rise times in control is spread in control, but less so in sucrose (Figure 5.10.F).

In contrast, focal application of sucrose to dendrites (Figure 5.11.B) increased the frequency of events of similar size as recorded in control conditions (n = 4) (Figure 5.8.A,C). The average mIPSC collected in control and in sucrose are of similar amplitude (19.5 pA in control and 20.2 pA in sucrose), however 10 - 90 % rise time was slightly reduced in sucrose (1.0 ms in control and 0.9 ms in sucrose) (Figure 5.11.D). Histogram plots of mIPSC amplitudes reflect these results with similar histogram distributions for peak amplitude in control and sucrose (Figure 5.11.E),

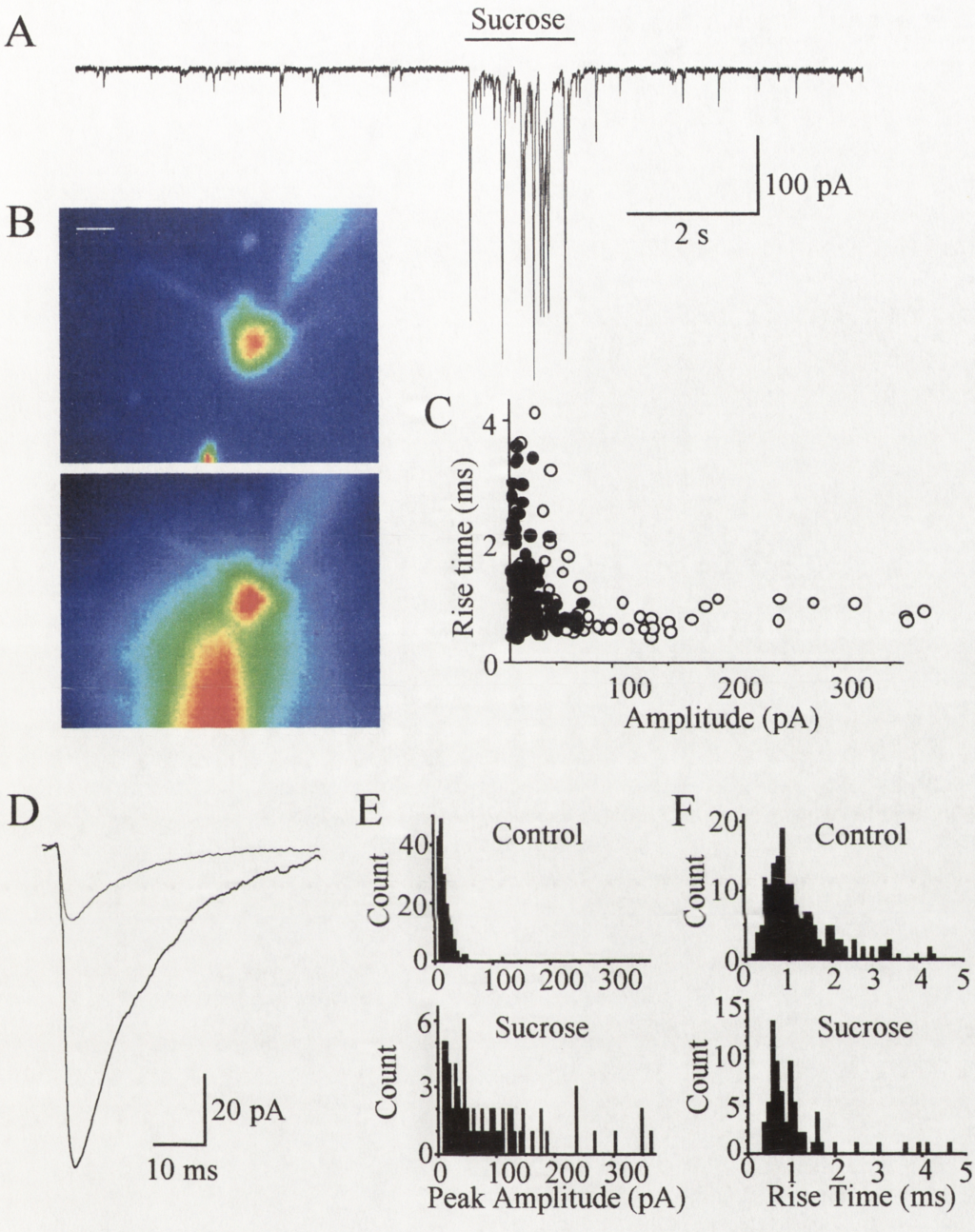

Figure 5.10. Focal somatic application of hypertonic sucrose.
A. Sucrose was applied under IR-visual guidence to the soma inducing a burst of mIPSCs of larger amplitude than those recorded in control. B. Lucifer yellow fluorescence ΔF indicating the extent of sucrose solution spreading (scale bar = 5 μM). C. Scatter plot of 10-90% rise time versus amplitude in control and during sucrose application. D. Average mIPSCs for events collected in control (thin line) and during sucrose application (thick line). Distribution of mIPSC amplitude (E.) and rise time (F.) in control and during sucrose application.

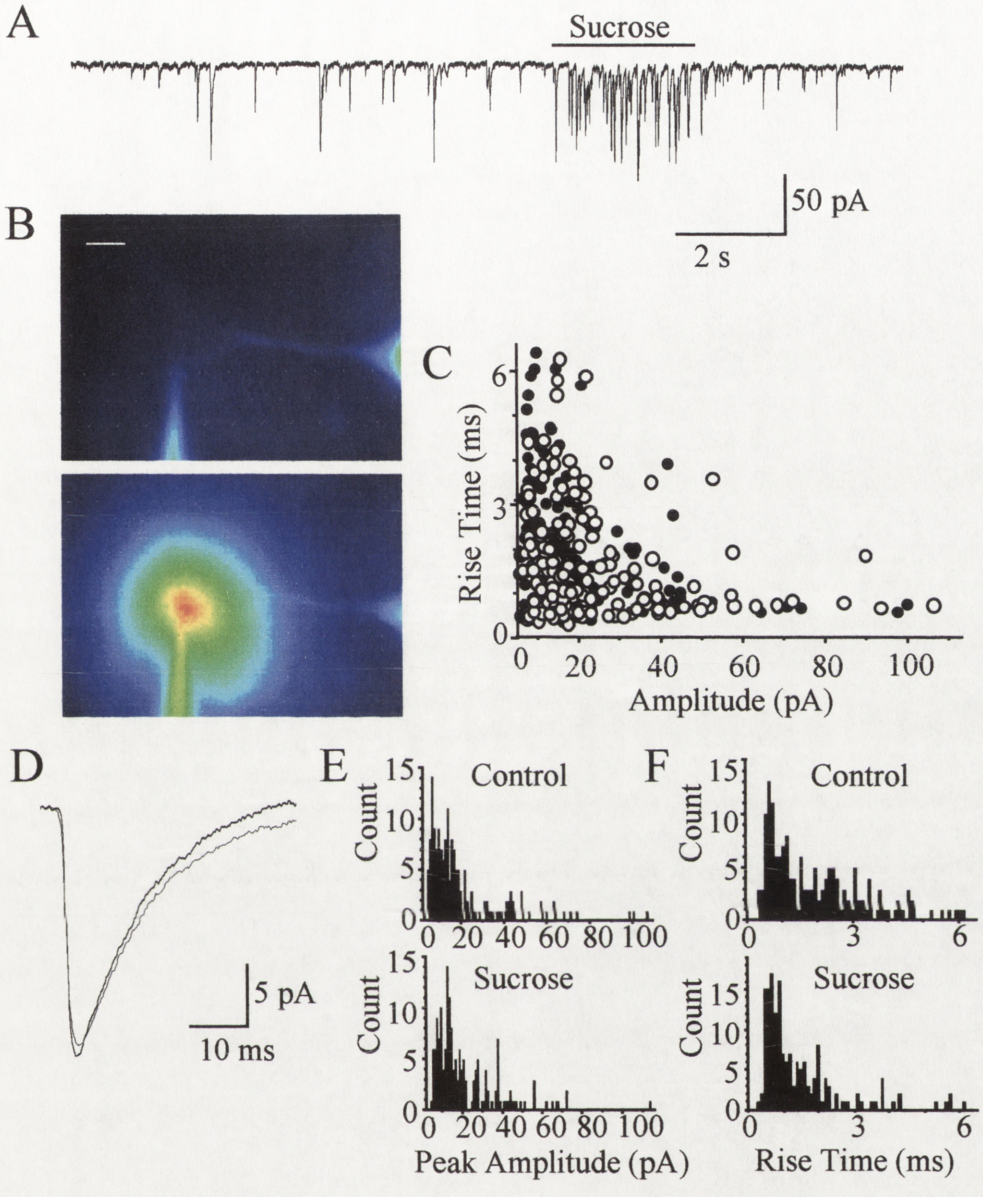

Figure 5.11. Focal dendritic application of hypertonic sucrose.
A. Sucrose was applied under IR-visual guidence to a region of dendrite inducing a burst of mIPSCs of similar amplitude than those recorded in control. B. Lucifer yellow fluorescence ΔF indicating the extent of sucrose solution spreading (scale bar = 5 μM). C. Scatter plot of 10-90% rise time versus amplitude in control and during sucrose application. D. Average mIPSCs for events collected in control (thin line) and during sucrose application (thick line). Distribution of mIPSC amplitude (E.) and rise time (F.) in control and during sucrose application.

but more slower rising events in control than in sucrose (Figure 5.11.F). This is probably a result of applying sucrose focally to an area of primary dendrite rather than to secondary and more distal dendritic regions, due to limitations of the visualisation technique used. The effects of cable filtering, evident in the spread of rise time of control population would not be evident or would be expected to be reduced by inducing mIPSCs selectively from this proximal dendritic region. The fact that the amplitudes of the events recorded in control and sucrose are the same, indicates that in both cases the smaller dendritic synapses were activated only.

These results indicate that the low frequency pure $GABA_A$ mIPSCs which have large amplitudes, occur at synapses on or very close to the soma of CeL neurones, whereas the predominant population of mixed receptor mIPSCs result from synapses localised dendritically.

5.2.5. Lateral stimulation activates dendritic mixed GABA receptor synapses

The IPSC described in the previous chapter were stimulated electrically in the ICM area lying between the CeL and the LA. These compound IPSCs were found to be partially blocked by bicuculline in all cases (n = 19), with an average block of 66.9 ± 3.3 % (Figure 4.7.), and by TPMPA in all cases (n = 6) with an average block of 36.8 ± 2 % (Figure 4.11.). The uniform block of the control mIPSC population (Figure 5.5.B) (when the pure $GABA_A$ mIPSCs of somatic synapses are seen very rarely or not at all) indicates that each individual synapse activated contains a proportion of both GABA receptors. To confirm this, the bicuculline and TPMPA sensitivity of small unitary minimal stimulation IPSC was examined. These minimal inputs were generated by incrementally increasing the electrical stimulus until an initial response is generated. The stimulus strength/response curves were generated (Figure 5.12.A) and the smallest consistent evoked response was used to examine the effects of the antagonists (excluding failures from the averages).

In all cases (8 minimal stimulation inputs in 7 neurones) a partial block of the laterally stimulated minimal IPSC was seen on addition of 10 μM bicuculline (Figure 5.12.B) and TPMPA. The average block by bicuculline was 71.1 ± 3.7 % of control peak amplitude ($p < 0.01$, n = 8) which is similar to the average block of the

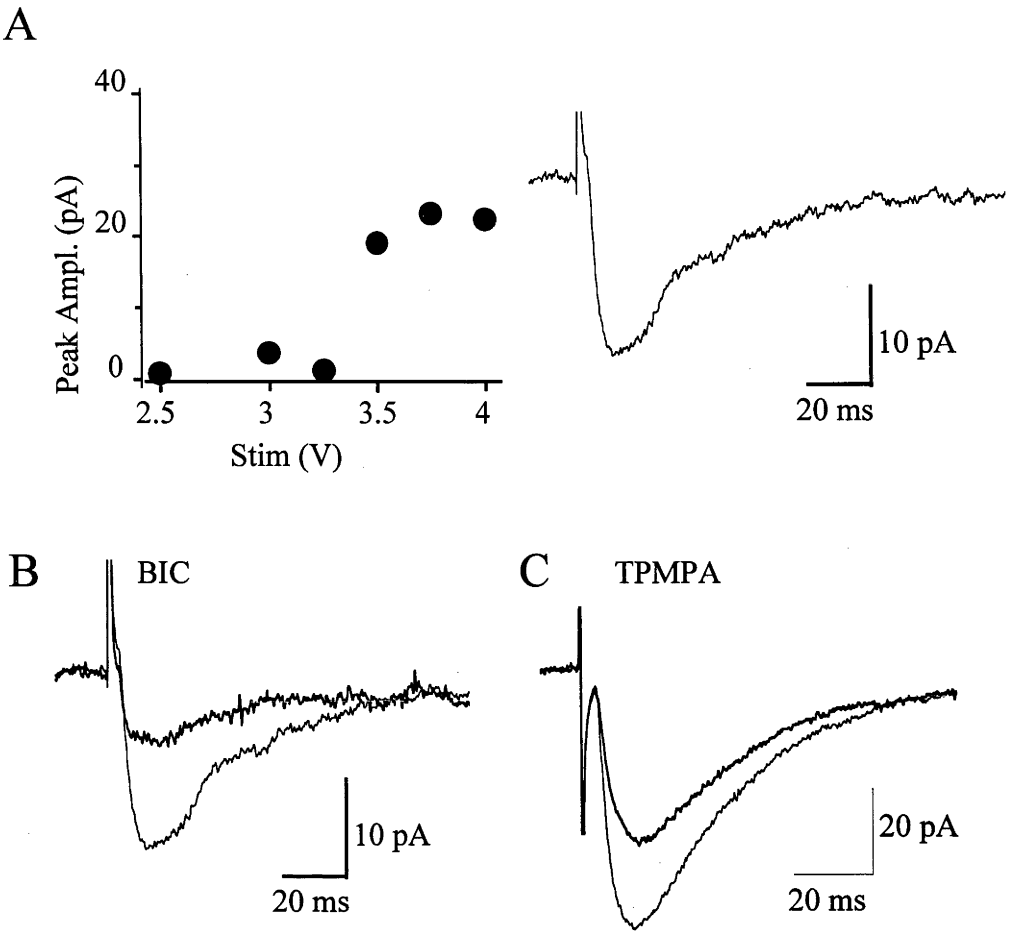

Figure 5.12. Laterally evoked minimal IPSCs are partially blocked by either bicuculline or TPMPA.

A. Stimulus response curve for stimulation of minimal evoked IPSCs in the ICM region lateral to the CeL (recorded in 2 mM kynurenic acid), and minimal input recorded with 3.5 V stimulus. Minimal stimulation IPSCs (average responses shown) are partially blocked by bicuculline (B.) or TPMPA (C.) (n = 8 and 4 respectively).

compound IPSC recorded previously. The TPMPA block of 46.9 ± 9.7 %, (n = 4, $p < 0.05$), was slightly higher than the block of the compound IPSC as a slightly higher concentration of TPMPA was used in the later experiments to ensure complete block of the bicuculline insensitive receptors (100 μM compared to 60 μM). These results indicated that these responses were partially mediated by both GABA receptors. Furthermore, the average amplitude of these minimal stimulation events (19.3 ± 3.8 pA, n = 10) was much smaller than the amplitudes of the mIPSCs induced by somatic sucrose application but not significantly different from the average control mIPSC without sucrose (20.4 ± 2.1 pA).

5.2.6. Medial stimulation activates somatic $GABA_A$ synapses

With the aim of identifying alternate inhibitory inputs into the CeL, electrical stimulation in various positions around the circumference of the Ce and in the CeM, were performed in the presence of kynurenic acid (2 mM). Monosynaptic IPSCs were stimulated with the stimulating electrodes place within the CeM and in the area medial to the CeM. Minimal stimulation responses generated here were larger and faster rising IPSC than those evoked by the lateral stimulation, as represented by the typical response shown in Figure 5.13.A. The average amplitude of the medial minimal stimulation response was 130.8 ± 13.4 pA and the rise time 1.7 ± 0.1ms (n = 10) compared to 19.3 ± 3.8 pA and 3.19 ± 0.4 ms for the lateral inputs (Figure 5.13. B,C). In contrast to the lateral responses, the medial minimal stimulation inputs were unaffected by TPMPA (1.1 ± 3.2 % inhibition, n = 3, Figure 5.13.D), but completely blocked by 10 μM bicuculline (98.3 ± 3 %, $p < 0.01$, n = 7, Figure 5.13.E). These results indicate that these evoked IPSC, like the fast rising spontaneous events induced by somatic application of sucrose, are also mediated by $GABA_A$ receptors only.

5.2.7. Effect of Diazepam on lateral and medial IPSC.

In the previous chapter, the effect of the 1,4-benzodiazepines diazepam and flurazepam was shown to be inhibitory on the bicuculline resistant receptors, while it positively modulated the $GABA_A$ receptors (Figure 4.15. and 4.16.). To compare the

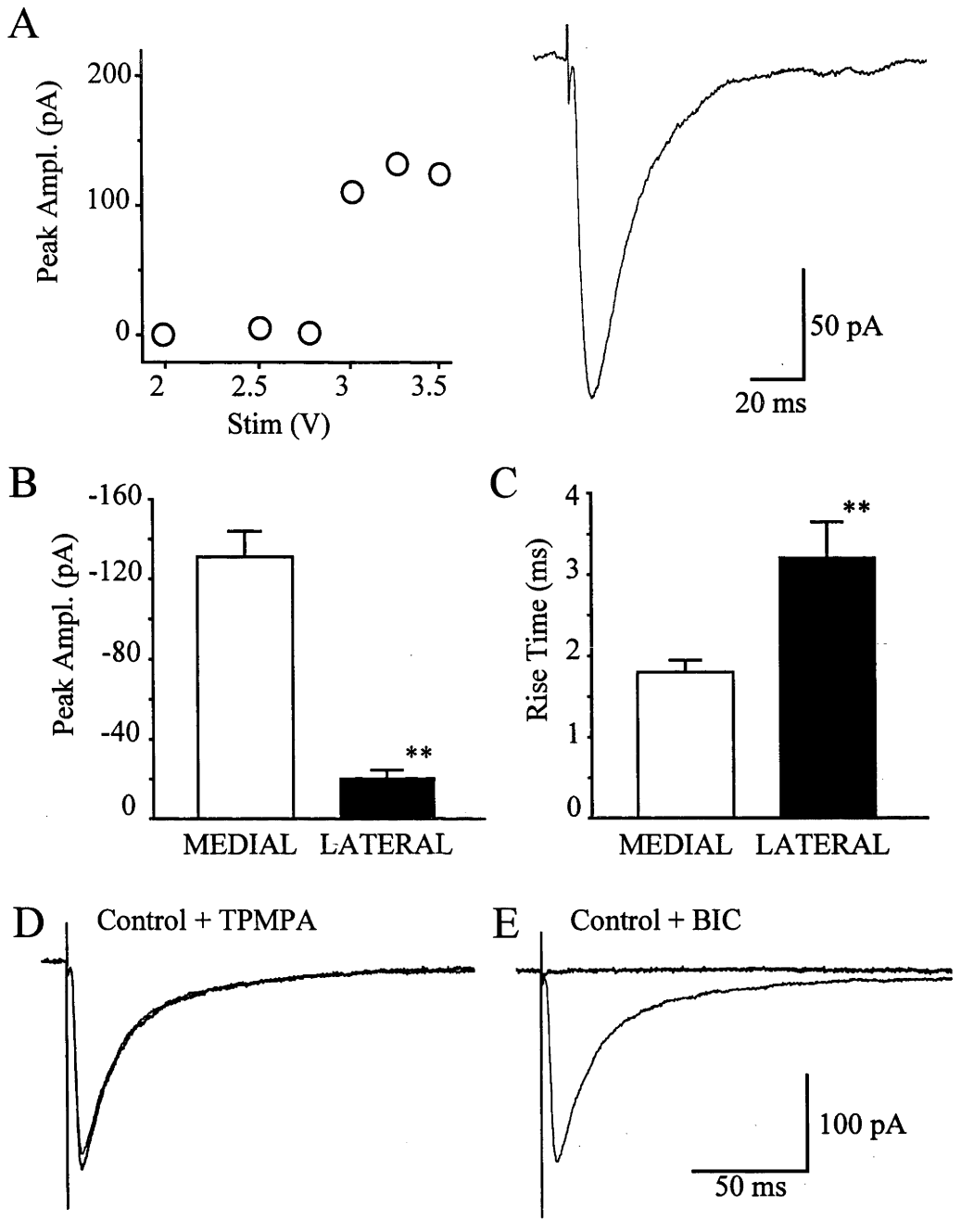

Figure 5.13. Medially evoked minimal IPSCs are larger and faster rising than those evoked laterally , and are blocked by bicuculline only.

A. Stimulus response curve for stimulation on the medial edge of the central amygdala, and the average of six medial minimal stimulation IPSCs stimulated (recorded in 2 mM kynurenic acid). Average amplitude (B.) and rise time (C.) of medially evoked minimal IPSCs compared to those evoked laterally.

D. Averages of six medially stimulated minimal IPSCs in control (2 mM kynurenic acid) and in TPMPA (100 μM). E. Averages of six medially stimulated minimal IPSCs in control and blocked by bicuculline (10 μM).

net effects of these drugs on laterally and medially inhibitory stimulated synapses, they were applied to minimal stimulation responses stimulated from both aspects.

Application of diazepam to the laterally stimulated minimal IPSC (Figure 5.14.A) resulted in a decrease in peak amplitude, but an increase in 1/2 peak width. The average effects for 5 cells being an 18.8 ± 7.5% decrease in amplitude and a 44.3 ± 16.2% ($p < 0.05$) increase in 1/2 peak width (Figure 5.14.C). In contrast, application of diazepam to the medially stimulated minimal IPSC (Figure 5.14.B) increased in both amplitude (13.7 ± 10.1%) and 1/2 peak width (39.7 ± 7.1%, $p < 0.01$, $n = 6$) (Figure 5.14.C).

These results are consistent with the medially stimulated IPSC being mediated by $GABA_A$ receptors only, which are positively modulated by diazepam, and the lateral IPSC being mediated by a mixed receptor population, which is both positively and negatively modulated, resulting in a net decrease in amplitude but an increase in 1/2 width of these IPSC.

5.3. DISCUSSION

5.3.1. Two types of inhibitory synapses on CeL neurones

These experiments indicate that the inhibitory synapses on CeL neurones fall into two catagories – pure $GABA_A$ and mixed GABA receptor synapses.

The mixed receptor synapses which express both the $GABA_A$ and bicuculline insensitive GABA receptors are partially blocked by either bicuculline or TPMPA with a consistent average contribution of the $GABA_A$ receptors of approximately 66% of the response. These synapses mediate the vast majority of the spontaneous inhibitory activity affecting the cell and afferents to these synapses can be stimulated electrically by in the ICM regions lateral to the Ce. The amplitude and bicuculline/TPMPA sensitivity of control mIPSCs is not significantly different from

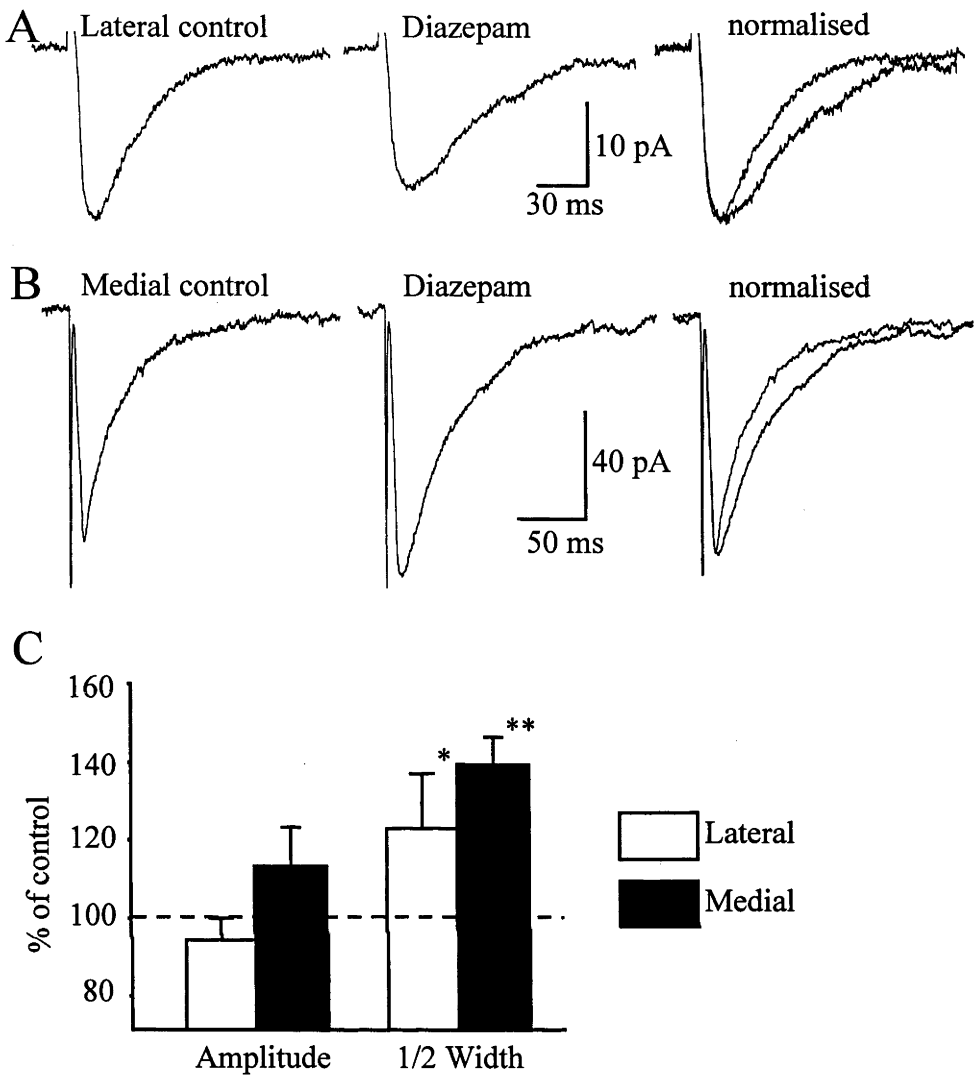

Figure 5.14. Diazepam reduces laterally evoked IPSCs but increases IPSCs evoked medially.

A. Typical laterally evoked minimal IPSCs in control and in diazepam (10 μM), and normalised (averages of six individual IPSCs shown). B. Typical medially evoked minimal IPSCs in control and in diazepam (10 μM), and normalised (averages of six individual IPSCs shown). C. Average effects of diazepam on laterally and medially evoked IPSC amplitude and 1/2 width ($p < 0.05$, $n = 6$ and $p < 0.01$, $n = 6$ respectively).

those of unitary IPSC responses to minimal stimulation of these lateral afferents, indicating that control mIPSCs are spontaneous activation of the same synapses. The rise time of spontaneous events and evoked responses from these synapses is slow indicating dendritic origin. This is further demonstrated by increased spontaneous activity of similar amplitude and rise time resulting from dendritic application of hypertonic sucrose. These synapses thus have been demonstrated as dendritic, mixed GABA receptor synapses, whose afferents arrive from the lateral aspect of the CeL and release spontaneously at high frequency.

The pure $GABA_A$ synapses were found to be activated at very low frequency in control conditions, but at higher frequency when hypertonic sucrose was applied to the somata of the CeL neurones. These mIPSCs, like the evoked IPSCs resultant from electrical stimulation on the medial aspect of the Ce or in the CeM, were completely bicuculline sensitive and TPMPA insensitive. Both the somatic sucrose induced mIPSCs and the medially evoked IPSCs were larger and faster rising than the dendritic mixed receptor IPSCs confirming the proximity of these synapses to the soma. Thus in summary, medially arriving afferents make somatic inhibitory synapses on CeL neurones producing large synaptic currents which are mediated by $GABA_A$ receptors only and activated at very low frequency in control conditions. Dendritic inhibitory synapses are mixed GABA receptor synapses which are made with local inhibitory interneurones. These inputs may be stimulated directly laterally in the ICM region, or by stimulation of a disynaptic circuit from the basolateral complex.

5.3.2. Two inhibitory inputs to CeL neurones

Axonal tracing studies have revealed several potential inputs, other than those from the basomedial amygdaloid complex, which may potentially form these somatic $GABA_A$ inhibitory synapses. Ascending projections from various brainstem and hypothalamic nuclei have been demonstrated to project heavily into all divisions of the Ce. A feedback inhibitory input from these ascending pathways may well provide powerful and acute inhibitory control over the output of the CeL to shut down CeL mediated effects on autononic pathways originating in the brainstem and hypothalamus. Tracing studies have shown that the passage of axons involved in

these ascending pathways enter into the amygdala via an area medial to the CeM, and dorsal to the medial nucleus of the amygdala, corresponding to the region between ansa lenticularis and the basal nucleus Meynert (Paxinos & Watson, 1986). This region corresponds to the region stimulated electrically in the medial stimulation somatic inhibitory synapses.

The afferents to the mixed receptor synapses were stimulated in the area corresponding to the location of the ICM, situated laterally to the Ce. These IPSC were also stimulated as a disynaptic input by stimulating in the BLA or LA (Figure 3.5.). Royer *et al* have shown in guinea pig that stimulation in the LA and BLA elicites a feed forward IPSC in CeM neurones from GABAergic ICM neurones which is partly bicuculline insensitive (Royer *et al.*, 1999). ICM neurones were also shown to projecting locally into the CeL (Royer *et al.*, 1999) which may similarly innervate the mixed receptor synapses on the CeL. Alternately, LA neurones have been shown to project to the CeL in cat (Smith & Paré, 1994) and the capsular region of the CeL in rat (Pitkänen *et al.*, 1995). It is thus, also possible that the stimulation in the ICM region may have stimulated GABAergic neurones on the lateral edge of the CeL itself (capsular region), though the comparatively light projection from the capsular area to the rest of the CeL shown by Jolkannen and Pitkanen (1998) would make this unlikely.

6. GENERAL DISCUSSION

The research embodied in this thesis has in the main part investigated the inhibitory transmission that occurs in the lateral division of central amygdala. From this research three notable findings have been made; CeL fast inhibitory transmission is mediated by two pharmacologically distinct subtypes of GABA receptors, these receptors are colocalised at dendritic synapses while somatic synapses contain $GABA_A$ receptors only, and medially arriving inhibitory inputs selectively activate these somatic synapses, whereas input from the lateral input activate the dendritic synapses.

The presence of a novel type of inhibitory response was first noticed by Nose *et al* (1991), who described strychnine sensitive IPSCs that were not blocked by bicuculline. While the presence of glycine receptors on CeL neurones was demonstrated, no evidence of their participation in the synaptic inhibitory currents was found. Rather, the bicuculline insensitive inhibitory currents presumably underlying the bicuculline insensitive IPSP reported by Nose *et al* (1991) were demonstrated to be mediated by a GABA receptor. This receptor subtype is distinct from typical $GABA_A$ receptors in that it is insensitive to low concentrations of bicuculline but sensitive to TPMPA, it is less sensitive to barbiturate modulation and insensitive to anaesthetic modulation, and it is negatively modified by classical 1,4-benzodiazepines. The central amygdala has been demonstrated to contain very high density of GABAergic cells, GABAergic terminals (Nitecka & Ben-Ari, 1987) and benzodiazepine binding sites (Niehoff & Kuhar, 1983). Furthermore, benzodiazepines have been used for some time to treat anxiety disorders which have been suggested to result from dysfunction of the amygdala (Davis, 1992; LeDoux, 1996). The presence in this structure of a high density of GABA receptors, which are not potentiated by these drugs but inhibited by them, may indicate that the action of these drugs is more complex than previously thought. Also, the isolation and identification of the GABA receptor subunit structure of these receptors may provide new insights into the action of allosteric modulators of GABA receptors and perhaps lead to the development of new therapeutic agents directed specifically at these receptors.

The segregation of $GABA_A$ receptor subtypes at particular postsynaptic sites has been demonstrated immunohistochemically (Fletcher *et al.*, 1998; Gustincich *et al.*, 1999; Nusser *et al.*, 1996), and more indirectly by examining the kinetic changes associated with post-translational modification of particular subunit combinations (Nusser *et al.*, 1999). $GABA_C$ receptors have also been demonstrated to be either localised at separate synapses to $GABA_A$ receptors in the retina (Fletcher *et al.*, 1998; Koulen *et al.*, 1998). The pharmacological demonstration of co-localisation of the two GABA receptor at dendritic synapses, but exclusion of the bicuculline resistant type at particular somatic synapses is further evidence that GABA receptor subcellular localisation is highly organized. The functional benefit of having multiple receptors with different regulatory or kinetic properties at particular synapses may provide a means for increasing or decreasing synaptic strength at these sites selectively. For instance, if a mixed synapse contained a receptor type that was inhibited by a naturally occurring allosteric modulator (such as a neuroactive steroid, a naturally occurring benzodiazepine site ligand (Medina *et al.*, 1993) or Zn^{2+} (Perez-Clausell *et al.*, 1989)) and one that was positively modulated, and another synapse contained only receptors that were positively modulated, the effect of such a modulator would be a net effect at the mixed synapse, depending on the receptor proportions, but positive at the single receptor synapse. Thus, this inhibitory pathway may be selectively enhanced by these agents over the pathway through the mixed synapse. Furthermore, increasing the proportion of the receptor inhibited by the allosteric modulator may provide a means for down-regulating the inhibition through this pathway when these modulators are present. The demonstration that applying benzodiazepines to the dendritic synapse IPSC activated by lateral stimulation reduced amplitude and increased decay less that that of the medially stimulated somatic synapses, which are also increased in amplitude, may illustrate this. Another possibility for mixed synapses is that one of the receptor types has higher sensitivity for GABA and/or exhibits less desensitisation (as is the case for ρ1 $GABA_C$ receptors), and that including more of this receptor in a mixed synapse may increase the sensitivity of these sites to low concentrations of GABA (perhaps increasing responses to GABA spillover), or to increase the duration of the inhibitory responses occurring at these sites (Sheilds *et al.*, 2000).

Finally, this research demonstrated that the CeL receives inhibitory input from the basolateral amygdaloid complex (albeit indirectly through the ICM neurones) and inhibitory input from a pathway that arrives medially. In the context of the known sources of innervation of these neurones, there are two likely candidates for the origin of these fibres. Firstly, the concept of the central extended amygdala includes an interconnected group of nuclei (the Ce, BNST and SI) which all receive similar afferents and project to similar effector regions in the brainstem and hypothalamus. The nature of the interconnections within the CEA has been described as an intrinsic system of GABAergic projections (Cassell *et al.*, 1999), which may integrate the output of the CEA. The second potential source of inhibition for these neurones may be the brainstem. These inputs may be reciprocal connections from the primary target areas of the Ce in the brainstem or alternatively they may be primary input arriving from the parabrachial nucleus as part of the nociception pathway. The latter pathway in particular may rapidly inhibit the locally projecting inhibitory network, causing rapid disinhibition of the projection neurones of the CEA. Such a pathway would require rapid and strong inhibition of these intrinsic GABAergic interneurones. Large inhibitory synapses on the soma or perhaps on the spine structures of the initial segments of the neurones would be ideally placed to mediate this rapid and complete inhibition. This pathway would be ideally placed to initiate protective arousal effects associated with unconditioned startle responses, which are independent of basolateral amygdaloid processing (Davis & Shi, 1999). Furthermore it has been suggested that the central amygdala may contribute to the plasticity that underlies the conditioned fear response, through the convergence of the unconditioned stimulus arriving from the parabrachial nucleus and conditioned stimulus pathways arriving via the thalamus, cortex and basolateral amygdaloid complex (Kapp *et al.*, 1992). Thus, convergence of excitation via the BLC and disinhibition within the CEA may provide a mechanism for conditioning.

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GABA Receptors Inhibited by Benzodiazepines Mediate Fast Inhibitory Transmission in the Central Amygdala

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The amygdala is intimately involved in emotional behavior, and its role in the generation of anxiety and conditioned fear is well known. Benzodiazepines, which are commonly used for the relief of anxiety, are thought to act by enhancing the action of the inhibitory transmitter GABA. We have examined the properties of GABA-mediated inhibition in the amygdala. Whole-cell recordings were made from neurons in the lateral division of the central amygdala. Application of GABA evoked a current that reversed at the chloride equilibrium potential. Application of the GABA antagonists bicuculline or SR95531 inhibited the GABA-evoked current in a manner consistent with two binding sites. Stimulation of afferents to neurons in the central amygdala evoked an IPSC that was mediated by the release of GABA. The $GABA_A$ receptor antagonists bicuculline and picrotoxin failed to completely block the IPSC. The bicuculline-resistant IPSC was chloride-selective and was unaffected by $GABA_B$-receptor antagonists. Furthermore, this current was insensitive to modulation by general anesthetics or barbiturates. In contrast to their actions at $GABA_A$ receptors, diazepam and flurazepam inhibited the bicuculline-resistant IPSC in a concentration-dependent manner. These effects were fully antagonized by the benzodiazepine site antagonist Ro15–1788. We conclude that a new type of ionotropic GABA receptor mediates fast inhibitory transmission in the central amygdala. This receptor may be a potential target for the development of new therapeutic strategies for anxiety disorders.

Key words: $GABA_C$; fear; anxiety; diazepam; bicuculline; amygdala

GABA is the major inhibitory transmitter in the mammalian CNS (Nicoll et al., 1989). As with many other types of receptor, two broad types of GABA receptor are recognized: ionotropic ligand-gated channels and metabotropic G-protein-coupled receptors. Ionotropic GABA receptors are further subdivided into the bicuculline-sensitive $GABA_A$ receptors (MacDonald and Olsen, 1994; Johnston, 1996a) and the bicuculline-insensitive $GABA_C$ receptors (Qian and Dowling, 1994; Bormann and Feigenspan, 1995). $GABA_A$ receptors gate a chloride ionophore and have modulatory binding sites for benzodiazepines, barbiturates, and anesthetics, all of which potentiate the response to GABA (MacDonald and Olsen, 1994; Johnston, 1996a). These receptors are potently inhibited by the competitive antagonists bicuculline and SR95531 and the plant alkaloid picrotoxin (Sieghart, 1995).

$GABA_A$ receptors are assembled from a large family of which fifteen members have so far been identified: 6α, 4β, 3γ, 1δ, and 1ϵ (Barnard et al., 1998). Heterologous expression of different subunits has shown that functional GABA receptors can form as homomers or as heteromultimers of different subunits. However, most $GABA_A$ receptors in the CNS are thought to contain both α and β subunits, with one or more of the γ, δ, or ϵ subunits (Barnard et al., 1998). The subunit combination of a particular GABA receptor determines its pharmacological properties (Costa, 1998; MacDonald and Olsen, 1994). For example, amplification of GABA action by benzodiazepines is only seen in receptors that contain one of $\alpha1$, $\alpha2$, $\alpha5$ subunits and either a $\gamma2$ or a $\gamma3$ subunit. Receptors that contain $\alpha4$, $\alpha6$, or $\gamma1$ are unaffected by benzodiazepines (MacDonald and Olsen, 1994; Costa, 1998).

$GABA_C$ receptors also gate a chloride channel, but they are not blocked by bicuculline or SR95531, and are markedly less sensitive to picrotoxin. They are also insensitive to modulation by benzodiazepines and barbiturates (Qian and Dowling, 1993; Bormann and Feigenspan, 1995; Johnston, 1996b). $GABA_C$ receptors are assembled from ρ subunits ($\rho1$, $\rho2$, $\rho3$), which share some homology with $GABA_A$ receptor subunits, but do not appear to coassemble with them. $GABA_C$ receptors have only clearly been demonstrated in the retina (Qian and Dowling, 1994; Enz et al., 1995). Bicuculline-resistant responses to GABA have been reported in several brain regions (Drew et al., 1984; Arakawa and Okada, 1988; Strata and Cherubini, 1994). However, the importance of these receptors outside of the retina has yet to be demonstrated.

The amygdala is intimately involved in emotional behavior, and its role in the generation of anxiety and conditioned fear is well known (Kluver and Bucy, 1939; LeDoux, 1995). Benzodiazepines, which are commonly used for the relief of anxiety, are thought to produce their therapeutic effect by enhancing the action of GABA (Tallman and Gallager, 1985; Costa and Guidotti, 1996). The action of benzodiazepines on GABA receptors within the amygdala is likely to be responsible for the anti-anxiety action of these agents because binding sites for benzodiazepines are present in the amygdala at high density (Niehoff and Kuhar, 1983; Richards and Möhler, 1984). In this study we have examined the properties of ionotropic GABA receptors in the central amygdala. We find that neurons in the central amygdala

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(Hamann et al., 1988). On average, 10 μM BIC blocked the GABA-activated current by 65 ± 6%, and 100 μM by 92 ± 3% (n = 11; Fig. 1*E*); 1 and 10 μM SR95531 blocked the iontophoretic current by 77 ± 6 and 93 ± 6% (n = 3), respectively. These results suggest that two types of ionotropic GABA receptor are present on CeL neurons. To rule out the possibility that the low sensitivity of the GABA response might be caused by inadequate access of the bath-applied antagonists to their site of action, we examined a block of iontophoretically applied glycine by the selective antagonist strychnine. Glycine activated a chloride-mediated current in all cells tested (data not shown). Strychnine blocked this current at a single, high-affinity site with an IC_{50} of 79 nM (Fig. 1*D*), close to the reported IC_{50} for strychnine in isolated cells and membrane patches (Shirasaki et al., 1991; Jonas et al., 1998). At 10 μM BIC, the contribution of the high-affinity BIC sites to the GABA response will be negligible. We therefore used this concentration of BIC to examine the properties of the BIC-resistant GABA response.

The relative insensitivity of the GABA response to BIC and SR95531 suggests that a $GABA_C$ like receptor might be present. $GABA_C$ receptors can be blocked by high concentrations of picrotoxin (Polenzani et al., 1991) and the selective antagonist TPMPA (Ragozzino et al., 1996). In confirmation of this, we found that the GABA response resistant to BIC was blocked by 88 ± 1% by 100 μM picrotoxin (n = 3) and by 73 ± 1% by 60 μM TPMPA (n = 3; Fig. 2). These results show that iontophoretically applied GABA activates two pharmacologically distinct receptors. One has a high affinity for BIC and SR95531, and represents activation of $GABA_A$ receptors, the other is relatively resistant to BIC and SR95531 but is antagonized by TPMPA.

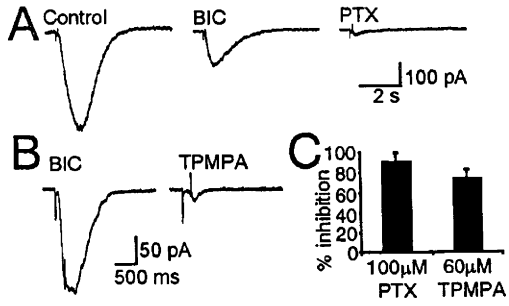

Figure 2. $GABA_C$-like receptors are present in the central amygdala. *A*, Responses to iontophoretically applied GABA recorded in control, 10 μM bicuculline, and 100 μM picrotoxin. *B*, The *trace* on the *left* is the response to iontophoretically applied GABA in the presence of 10 μM BIC. This current is blocked by application of the selective $GABA_C$ antagonist TPMPA (60 μM). *C*, Summary data showing the average reduction of the BIC-resistant response by picrotoxin and TPMPA.

We next asked if these two types of GABA receptor were also activated by synaptically released GABA. Stimulation of local afferents in the presence of glutamatergic antagonists evoked an IPSC that reversed near the chloride equilibrium potential (Fig. 3*A*,*B*), showing that it is a chloride-selective current. Application

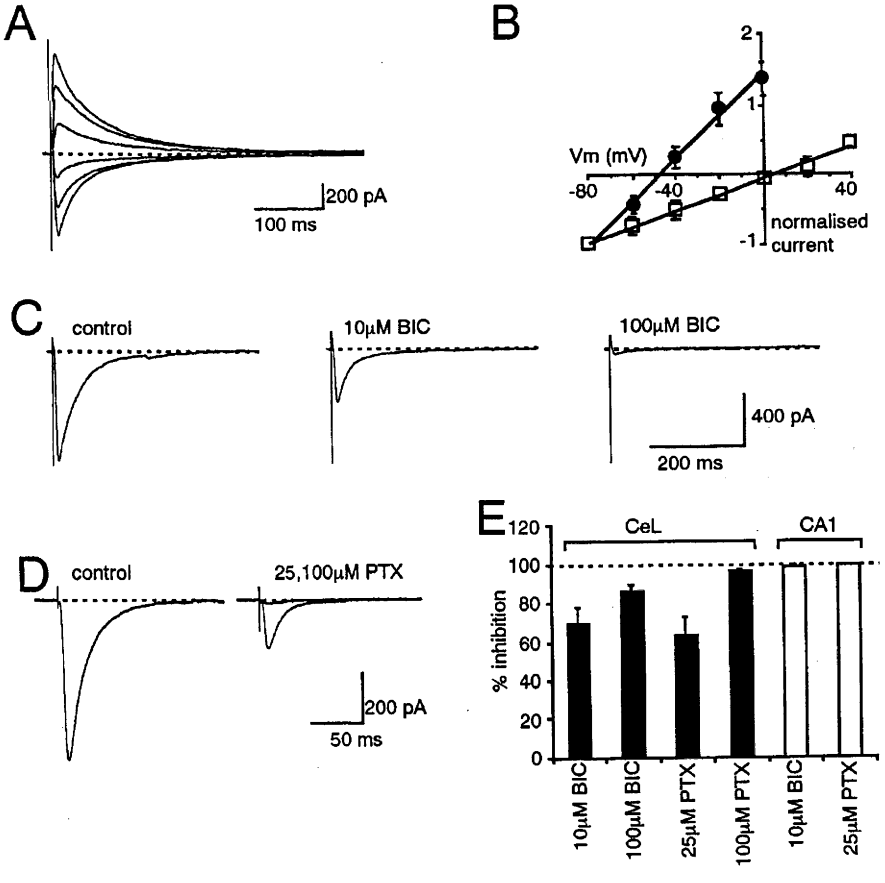

Figure 3. GABAergic inhibitory synaptic currents in the central amygdala are not blocked by low doses of bicuculline or picrotoxin. *A*, Synaptic currents in response to local electrical stimulation in the presence of blockers of glutamatergic receptors (see Materials and Methods) recorded at membrane potentials of −80, −60, −40, −20, 0, and 10 mV using low-chloride internal solution. *B*, Normalized current–voltage relationships for synaptic currents recorded using low-chloride (*closed circles*) (n = 4) and high-chloride internal solutions (*open squares*) (n = 2). The reversal potential in low-chloride internal was −52 mV, whereas in high-internal chloride it was 0 mV. *C*, Average CeL IPSCs recorded in control, in 10 μM bicuculline, and in 100 μM bicuculline. The IPSC was blocked to 67 ± 3% of control by 10 μM and 87 ± 3% in 100 μM BIC. *D*, IPSCs recorded in control, in 25 μM picrotoxin (*PTX*), and 100 μM PTX. IPSC amplitude was blocked by 64 ± 0% in 25 μM PTX. *E*, Summary of effects of antagonists bicuculline and picrotoxin on IPSC peak amplitude recorded from CeL neurons (*filled columns*) or from CA1 pyramidal neurons (*open columns*) (n = 5, 3, 3, 7, 5, and 4, respectively).

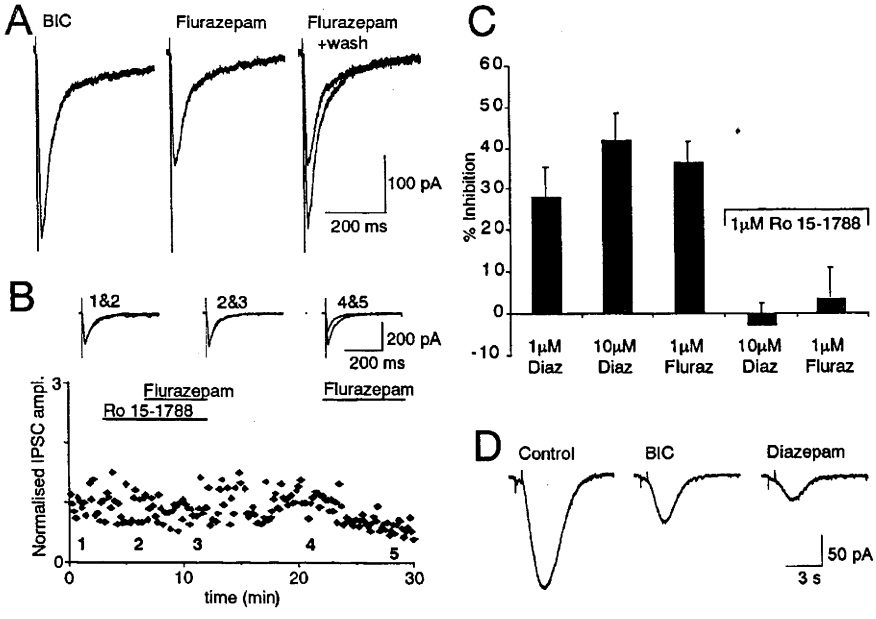

Figure 6. Diazepam and flurazepam inhibit $GABA_C$ receptors in the central amygdala. *A*, The effect of flurazepam on the BIC-resistant IPSC. Flurazepam (1 μM) reduced the peak amplitude (37 ± 5% of control; $n = 9$) with no effect on the kinetics of the current. *B*, The benzodiazepine site antagonist Ro15–1788 (1 μM) has no effect on IPSC amplitude but blocks the inhibitory effect of flurazepam. After washout of Ro15–1788, a second application of flurazepam now inhibits the IPSC. Individual records taken from the times indicated are shown above. *C*, Summary data showing the effects of flurazepam and diazepam on BIC-resistant IPSCs in control conditions ($n = 9$) and Ro 15–1788 ($n = 3$). *D*, The effects of benzodiazepines are postsynaptic because diazepam (10 μM) blocks the bicuculline-resistant response to iontophoretically applied GABA by 27 ± 8% ($n = 5$).

duced by 21.2 ± 7.4% ($p = 0.16$), and half-width increased by 32 ± 16% ($n = 3$; $p = 0.07$; Fig. 5*E,F*).

The 1,4-benzodiazepines act as positive modulators of some $GABA_A$ receptors (MacDonald and Olsen, 1994; Costa, 1998) by increasing the affinity of the receptor for GABA (Lavoie and Twyman, 1996), whereas $GABA_C$ receptors are insensitive to these agents. In the CeL, flurazepam (1 μM) reduced the amplitude of the BIC-resistant IPSC by 37 ± 5% ($n = 9$; Fig. 6*A*). Diazepam (1 μM), another 1,4 benzodiazepine, reduced the amplitude of the BIC-resistant IPSC by 28 ± 7%, and 10 μM diazepam reduced it by 42 ± 5% ($n = 5$; Fig. 6*C*). This effect was fully antagonized by the benzodiazepine receptor antagonist Ro 15–1788 (Hunkeler et al., 1981) (Fig. 6*C*), showing that it was not a nonspecific action of these benzodiazepines. There was no effect on the kinetics of the IPSC with either diazepam or flurazepam (Fig. 6*A,C*). To confirm that the effects of the benzodiazepines on IPSC amplitude were caused by their postsynaptic actions on GABA receptors, we tested the action of diazepam on iotophoretically applied GABA. Diazepam (10 μM) reduced the amplitude of the BIC-resistant GABA-evoked current by 27 ± 8% ($n = 5$; Fig. 6*D*), showing that the effects of diazepam are postsynaptic.

We performed two control experiments to ensure that the benzodiazepines were active at $GABA_A$ receptors in our hands. First, we checked the action of these drugs on $GABA_A$ synapses recorded from CA1 neurons in the hippocampus. Diazepam (1 μM) increased the amplitude of hippocampal $GABA_A$ receptor-mediated IPSCs by 34 ± 16% and its half decay by 17 ± 12% ($n = 5$), and at 10 μM, the IPSC amplitude and half width increased by 41 ± 16 and 28 ± 18%, respectively (data not shown). These effects are typical of the actions of benzodiazepines at $GABA_A$ synapses (Otis and Mody, 1992; Zhang et al., 1993). Second, we isolated the $GABA_A$-mediated IPSC in CeL neurons by performing experiments in the presence of TPMPA. TPMPA (60 μM) blocked the control IPSC by 27 ± 2% ($n = 4$). In the presence of TPMPA, bicuculline (10 μM) blocked the IPSC (96 ± 1% of control; Fig. 7*A*), confirming it was caused by activation of $GABA_A$ receptors. Application of flurazepam in the presence of TPMPA had no effect on the peak amplitude but increased the half width of the IPSC by 121 ± 5% ($n = 3$; Fig. 7*B*), showing that $GABA_A$ receptors that contribute to the IPSC have a typical pharmacology.

DISCUSSION

We have shown that in the CeL, both exogenously applied GABA and synaptically released GABA activate two types of ionotropic GABA receptor. One is a classical $GABA_A$ receptor, inhibited by BIC and positively modulated by benzodiazepines. The other is relatively insensitive to the classical $GABA_A$ receptor antagonists bicuculline and picrotoxin. This BIC-insensitive response is blocked by the $GABA_C$ antagonist TPMPA. Furthermore, like $GABA_C$ receptors, the BIC-insensitive component is not affected

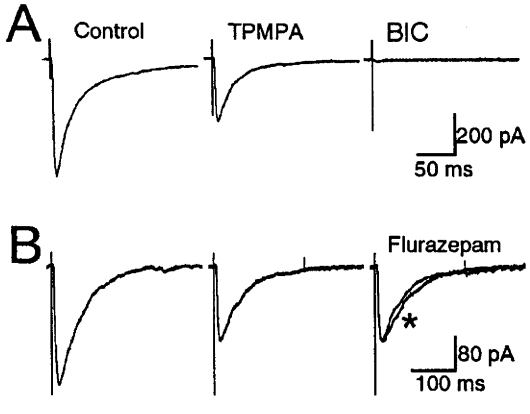

Figure 7. Pharmacology of $GABA_A$ receptors in the CeL. $GABA_A$ receptors were isolated by blocking $GABA_C$ receptors with TPMPA (60 μM). *A*, The IPSC remaining in the presence of TPMPA is effectively inhibited by 10 μM bicuculline. *B*, The $GABA_A$ receptor-mediated IPSC is positively modulated by benzodiazepines. Flurazepam had no effect on peak amplitude but increased the half width by 121 ± 5%.

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